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BACTERIA FERMENTING LACTOSE and THEIR SIGNIFICANCE IN WATER ANALYSIS

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By

MAX LEVINE

BULLETIN 62

ENGINEERING EXPERIMENT STATION

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BACTERIA FERMENTING LACTOSE
and
THEIR SIGNIFICANCE IN WATER ANALYSIS

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I. CHARACTERISTICS OF THE COLON GROUP OF BACTERIA*

The bacteriological analysis of water is an indirect and quantitative one. Specific pathogenic organisms are not sought nor are they likely to be detected even in a dangerous water. It devolves upon the analyst to interpret his findings and particular emphasis is placed on the determination of the presence of the colon group. The investigator and analyst should therefore be thoroughly acquainted with the characteristics, peculiarities, and idiosyncrasies of the organisms in the group, particularly with reference to their distribution, viability, and differential reactions.

Bacterium coli was first isolated by Emmerich from the feces of a cholera patient in 1884. It was soon recognized as a normal inhabitant of the intestinal tract of man and of other animals. For the past three decades the colon group of bacteria has been extensively studied by bacteriologists and sanitarians especially those interested in water supply and purification. Probably as much work has been done on this as on any other group of bacteria but there is not as yet an absolute agreement as to the limitations of the group.

Limitations of the Group. In the 1905 report of the Committee on Standard Methods for Water Analysis of the American Public Health Association a series of tests were described which were supposedly characteristic of the colon group. These tests included morphology, motility, fermentation of glucose, coagulation of milk, production of indol, reduction of nitrates, and gelatin liquefaction. Many of these reactions, as gelatin liquefaction, nitrate reduction, and indol formation, require a long incubation period. If the recommendations of the committee were adhered to, it would take at least nine days to justify the inclusion of an organism in the colon group. This was found to be very impractical and the tendency has been to simplify the preliminary tests necessary to place an organism in this category.

The 1917 and the 1920 Standard Methods of Water Analysis define the colon group as follows: "It is recommended that the *Bact. coli* group be considered as including all non-spore-forming bacilli which ferment lactose with gas formation and grow ærobically on standard solid media."

This characterization is concurred in by Winslow who defines the colon group as including all ærobic non-spore forming bacilli which produce acid and gas in glucose and lactose media.

Hauser modifies the definition somewhat by excluding gelatin liquefiers.

Other investigators are inclined to extend the colon group to include spore forming organisms which are capable of fermenting lactose with gas production and which grow ærobically on solid media. A few such strains have been recently encountered in the routine examination of water. Perry and Monfort include such spore forming types as members of the colon group. The statement of Löhnis and Smith, who have made studies

*In conformity with the recommendations of the committee on nomenclature of the Society of American Bacteriologists the colon group is considered in the genus **Bacterium.**

on the life cycle of bacteria, that a single species of *Azotobacter* may pass through as many as twelve to fourteen morphological forms including spores, is not particularly relevant with respect to *Bact. coli*, the life cycle of which has been carefully studied by Kellerman and Scales who note specifically that spores were not observed. It is conceivable that unfavorable environment may lead to spore formation by members of the colon-ærogenes group but the writer has never encountered nor heard of such a transformation, although he has observed a large number of cultures kept under various unfavorable conditions, nor does he anticipate such a fundamental and radical change in form.

These spore forming, ærobic, lactose fermenters confuse the ordinary tests for *Bact. coli* and must be taken into consideration in interpreting water analyses, just as it is essential to differentiate the anærobic spore forming lactose fermenters which confuse the presumptive test for *Bact. coli*, but there is no logical reason nor justification for placing them in the colon group.

Clark and Lubs raise the question as to the reliability of lactose fermentation as a primary criterion. They say, "If a fundamental cultural requirement of the members of the colon-ærogenes family is that it shall ferment lactose, there is imposed the same sort of requirement for the characterization of a whole family as is imposed by the MacConkey scheme when groups within the family are separated on the basis of the fermentation of another sugar, sucrose."

They point out that the fermentation of sucrose, which was formerly employed to subdivide the colon group into species and varieties (MacConkey's scheme), has been found less desirable than differentation on more recently devised tests, such as the gas ratio, the methyl-red reaction, and the Voges Proskauer test. They raise the question as to whether it is not possible that in the near future a test may be discovered which will supplant lactose fermentation as the salient and fundamental requirement for the whole colon-ærogenes family. We may well agree with Clark and Lubs that the future holds out to us promises of improved differential tests but we do not feel that, in consequence, we shall not utilize such means as are now available. Surely the classification of the organisms of this group to the best of our abiltiy on tests which are now known and used, would simplify a reclassification when these more fundamental, and we hope more reliable reactions of the future are brought out. The fact remains that the fermentation of lactose has been successfully employed for the separation of the non-pathogenic colon group from the disease producing para-typhoid and typhoid groups. Until a test is developed which will adequately supplant this, the fermentation of lactose with acid and gas production is considered a convenient and reasonably reliable criterion for members of the colon group of bacteria.

The colon group will therefore be considered to include non-sporing, Gram negative bacilli which ferment lactose with the production of acid and gas and which are capable of growing ærobically. The statement

frequently made, that the group consists of ærobes which ferment lactose is somewhat confusing, for the characteristic fermentation of lactose (gas formation) is determined under anærobic conditions.

DIFFERENTIAL TESTS

The colon group, as defined, is a large and complex one including a number of closely related but distinct species and varieties. The question arises whether all the members are of equal sanitary significance or whether some may not be more intimately associated with animal or particularly with human pollution thereby becoming of special significance in the interpretation of water or other examinations. It may be of practical value to distinguish and classify these different types and, if possible, to correlate them with their habitat. For these purposes a large number of tests have been employed.

The more important reactions to be considered are:

Coagulation of milk.
Gelatin liquefaction.
Production of indol.
Motility.
Fermentation of carbohydrates.
Production of acetyl-methyl-carbinol. (Voges Proskauer reaction).
Uric acid test.
Methyl red test.

Coagulation of milk. The test for the determination of the coagulation of milk is made by inoculating litmus or brom cresol purple milk which is then incubated at the body temperature for 48 hours. Acid is formed, some gas develops, and coagulation usually takes place in this time. If the milk has not been clotted the tube is immersed in boiling water for a few minutes or brought to a boil over a flame and if this treatment induces coagulation the reaction is considered as positive. If the milk has been over sterilized in the autoclave, coagulation does not take place as readily as when the medium is sterilized by the intermittent method in the Arnold. There is no digestion of the curd and the whey, if present, is clear. The litmus may be reduced by some strains. The milk reaction has been found very valuable in identification of the colon group.

Gelatin liquefaction. The liquefaction of gelatin is an important test for the differentiation of colon species. Studies by Gligler and by Johnson and Levine indicate that this test is well correlated with motility and fermentation of glycerol. Unfortunately the liquefaction of gelatin is difficult to determine. The period of incubation usually employed is fourteen days at 20 degrees C. Gage and Phelps, and later Johnson and Levine pointed out that the proportion of liquefiers recognized varies with the period of incubation as may be seen from Table 1.

Among 202 strains studied by Johnson and Levine, 106 (52%) were gelatin liquefiers after 34 days incubation but only 31 (15.3%) showed this reaction in 13 days.

TABLE I. LIQUEFACTION OF GELATIN BY 202 COLON STRAINS FROM SOIL.

Incubation days	Cultures liquefied	% of total liquefiers
2	17	16.0
7	17	16.0
13	31	29.2
20	38	35.8
27	61	57.5
34	106	100.0

The long incubation period necessary makes this test an inconvenient one for practical work and in some laboratories a modification has been introduced employing 37° C. for 48 hours. This of course liquefies the gelatin and so to determine whether a physiological decomposition has taken place the tubes are immersed in ice water until control tubes solidify. Inoculated tubes which remain liquid are regarded as having been peptonized by the action of the organism in question.

Indol. The production of indol from peptone is very extensively determined particularly in England where indol formers are regarded as "typical" *Bact. coli.* The test is usually carried out in the following manner:

One percent peptone water cultures are incubated for four or five days at body temperature. The culture is acidified with 1 c. c. of a ten percent solution of sulphuric acid and then 1 c. c. of a 1-5,000 potassium nitrite is added so as to form a layer on the surface. After a period varying from a few minutes to an hour a red ring will develop at the junction of the nitrite and acidified peptone culture if indol is present. This is known as the Salkowfsky test.

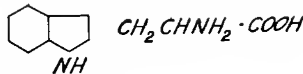
A more delicate reaction is obtained by the Ehrlich test which is performed thus:

To the culture add 3 c. c. para-dimethyl-amido-benzaldehyde and 3 c. c. of a saturated solution of potassium persulphate. Presence of indol is indicated by the production of a red coloration.

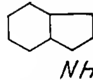
The significance and value of the test has been much in dispute. Howe found it to be but slightly correlated with other characteristics and consequently regarded it to be of little diagnostic value. Castellani and Chalmers on the other hand, consider indol of fundamental importance in classification of colon-like forms, and Houston believes it to be of particular significance for distinguishing the "typical" or "excretal" from the "atypical" *Bact. coli.* Levine found that among members of the colon group which were of intestinal origin, 91 percent formed indol, whereas of those obtained from soil only 37.3 percent were indol positive.

The constancy of the reaction has been questioned. Smirnow reported that subjection of *Bact. coli.* to the action of carbolic acid induces a loss of the ability to produce indol but that this character is regained after several sub-cultures in nutrient broth.

The source of indol in culture media is the amino acid tryptophane,



which is decomposed with the liberation of indol.



Many of the irregularities reported are undoubtedly due to varying quantities of tryptophane in the media employed and these may be eliminated by use of tryptophane broth as suggested by Kligler.

Motility. Motility may be determined either by the hanging drop method or by the use of a semi-solid medium such as Hesse agar. In the latter non-motile organisms grow along the line of inoculation with very little diffusion into the medium whereas the motile organisms grow rapidly away from the line of inoculation producing a distinct turbid zone of several millimeters, in 6 to 12 hours, which may easily be observed with the naked eye.

There is considerable disagreement as to the value of motility as an index and differential test for members of the colon group. There are undoubtedly both motile and non-motile colon bacilli in the intestinal tract of man. The character seems to be quite variable as a number of preliminary cultures are sometimes required to make motility evident and McWeeney has reported that some strains were motile at 20 degrees and not at 37 degrees C.

The statement that motile forms are characteristic of the human intestine appears to be in error as Stocklin (quoted by McWeeney) observed 116 non-motile strains among 300 colon bacilli from feces. Levine found only 32 percent of 25 cultures from man to be motile and only 20 percent of 30 cultures from raw and septic sewage whereas colon strains obtained from animals were almost always motile (sheep 77.3%, cow 80.0%, pig 93.7%, and horse 100%). It should be noted that these results were obtained with the use of a semi-solid agar (nutrient agar containing 0.5% agar).

The relation of motility as determined by the hanging drop and semi-solid media has recently been studied by Chen and Rettger with results indicated in Table II.

It appears that for the true *Bact. coli* there is excellent correlation in the two methods of motility determination. Out of a total of 173 cultures examined, 119 strains were found to be motile by the hanging drop method and 121 with the semi-solid agar medium. With the *Bact. aerogenes* strains, however, out of 477 cultures observed, 122 were motile by the hanging drop and only 75 by the agar method. It would seem that for the aerogenes types observation of motility in semi-solid media is undesireable.

TABLE II. RELATION OF HANGING DROP AND SEMI-SOLID AGAR FOR DETERMINATION OF MOTILITY.

(After Chen and Rettger, 1920)

Type of Organism	Hanging drop		Semi-solid agar (Hesse)		Number Examined
	Motile	Non Motile	Motile	Non Motile	
Bact. coli	119	54	121	52	173
Bact. aerogenes	122	325	75	372	447

Levine, however, in a study of 151 strains of the aerogenes-cloacae group, found an excellent correlation between motility, as determined in semi-solid medium, gelatin liquefaction and starch fermentation. Thus of 89 motile organisms 81 (91.0%) liquefied gelatin and only 4 (4.5%) fermented starch; whereas among 62 non-motile organisms only 2 (3.2%) liquefied gelatin while 61 (98.5%) fermented starch. A test which correlates so well with other characters is probably of differential value, and, although it is not recommended at present for routine work, it may be of significance and should be included in investigational studies.

MacConkey recommends that motility be observed in six hour cultures using dark field illumination. Castellani and Chalmers also employ motility as an important differential criterion.

Fermentation of carbohydrates. The carbohydrates which have been most commonly employed in the study of the colon group are listed below:

Monosaccharids. Glucose, levulose, and galactose.
Disaccharids. Lactose, sucrose, and maltose.
Trisaccharid. Raffinose.
Polysaccharids. Starch, inulin, and glycogen.
Alcohols. Glycerol, mannitol, dulcitol, and adonitol.
Glucoside. Salicin.

The media for fermentation tests generally consist of peptone water or broth containing one percent of the test substance. Incubation is at the body temperature for 48 hours and a positive reaction is indicated by gas production. If desired, litmus, brom cresol purple, neutral red, or the Andrade indicator may be added to the medium to observe acid formation.

Kligler suggests that quantitive acid-production be substituted for gas-formation as an index of fermentation. He points out that in standard meat-infusion sugar-freed carbohydrate broth media there is a rather sharp dividing line between acid-producers and nonacid-producers at 1.5 percent normal acid and that quantitive gas-production is variable and unreliable. Although quantitive gas-formation as ordinarly determined in the Smith or Durham tube is markedly inconstant and therefore of little

value, the fact that gas is produced at all may, nevertheless be of considerable significance. If a culture is inoculated into sugar broth and gas is formed, while no gas is produced in plain broth, the organism would most certainly be regarded as a fermenter of the test sugar irrespective of whether more or less than 1.5 percent acid is formed.

The low titer might be due to a secondary alkali-production which masks the acid, as suggested by Rogers. It has been repeatedly observed by the author that *Bact. aerogenes* in peptone dipotassium-phosphate solution, containing one percent or two percent glucose, may be acid to methyl red after 24 hours' incubation but alkaline after a period of 48 to 96 hours at 37 degrees C.

Rogers, Clark, and Evans also determined titratable acid and selected one percent normal acid as the point of demarcation between fermenters and non-fermenters but they point out the possible errors in acid-determination and give precedence to gas-formation, if positive.

The author's observations are that with peptone water as a base and one percent of the test carbohydrates, nonfermenters rarely produced as much as 0.2 percent normal acid.

At what point on the acid scale are fermenters to be differentiated from nonfermenters? There is considerable disagreement as to the maximal amount of acid formed by *Bact. coli.* Kligler, using meat infusion media, often obtained titers of four percent normal acid or more and similar results have been recorded by Rogers and others. Browne, however, using Liebig's meat-extract media, states that the limiting acidity for *Bact. coli* is 2.4% normal acid as determined by titration with phenolphthalein. Winslow and Walker determined acid-production in 12 substances by *Bact. coli.* The maximal acidity observed was 0.45 c. c. N/20 NaOH to the cubic centimeter of culture medium, or 2.25 percent normal acid.

The writer's experience, with peptone water as the basic medium, is in entire accord with Winslow and Walker, and with Browne. Of more than 2500 titrations, none showed over 2.4 per cent normal acid.

The difference in acid-production observed by various investigators is due to differences in the composition of the media employed. It is now well established that more acid is formed in meat-infusion broth than in beef-extract broth. In media containing much phosphates, as yeast water, even more acid is formed than in meat infusion broth.

Acid-production should not be given precedence over gas-formation. They may be independent characters. If, however, after careful studies, it appears that there is a marked correlation between quantitative acid-production and qualitative gas-formation, then it may be feasible to supplement, if not substitute, the gas test by the acid test. In that event, **the line of demarkation between fermenters and nonfermenters would have to be determined for the medium employed.**

Table III. shows the relation of gas-production to the amount of acid formed from sucrose, raffinose, ducitol, glycerol, and salicin in peptone water. Other test substances were observed but are not indicated because they were invariably fermented with production of gas.

TABLE III. RELATIONSHIP BETWEEN QAUNTITATIVE ACID-PRODUCTION AND GAS-FORMATION BY COLON GROUP.

Test Substance	Gas	Strain	Percentage of normal acid				
			0-0.19	0.20-0.39	0.40-0.59	0.60-0.79	0.80 or more
Sucrose	+	No.	0	0	8	48	19
		%			10.6	64.0	25.4
	−	No.	79	1	0	0	0
		%	98.8	1.2			
Raffinose	+	No.	1	0	2	18	58
		%	1.3		2.5	22.8	73.4
	−	No.	72	1	2	0	2
		%	93.5	1.3	2.6		2.6
Dulcitol	+	No.	0	2	5	23	37
		%		3.0	7.5	34.3	55.2
	−	No.	86	1	1	0	0
		%	97.8	1.1	1.1		
Salicin	+	No.	0	0	1	19	82
		%			10	18.6	80.4
	−	No.	43	1	3	6	1
		%	79.7	1.8	5.6	11.1	1.8
Glycerol	+	No.	0	0	16	61	41
		%			13.6	51.7	34.7
	−	No.	4	5	23	5	1
		%	10.5	13.2	60.5	13.2	2.6

It will be noted that acid-production in sucrose, dulcitol, and raffinose is well correlated with the presence or absence of gas. With salicin the correlation is not so marked, while with glycerol the line of demarcation between gas-formers and non-gas-formers, as indicated by the quantity of acid produced, is very indistinct. The substitution of quantitative acid-production for gas-formation would therefore be particularly undesirable when working with glycerol.

These results are well in accord with those of Winslow and Walker, who observe: "Gas-formation coincided with acidity except in the case of dextrin."

All investigators are agreed that members of the colon group normally ferment the monosaccharids with production of both acid and gas. Very detailed studies have been carried out on the products of the fermenattion of glucose, particularly the gas ratio and the H^{+}ion concentration. These careful observations have yielded very fruitful results.

The disaccharid lactose is of course fermented by all members of the group and maltose is also attacked. Sucrose is practically always decomposed by strains obtained from the soil, from grains, or from animal feces, but less frequently by strains isolated from human dejecta or sewage. The fermentation of sucrose has been recognized by many investigators as a convenient and important character for subdivision. It is the primary character in the MacConkey classification, it is employed by Jackson, and has been recognized by all the more recent investigators of the colon group as a most important and convenient differential characteristic.

The trisaccharid raffinose is fermented by practically all strains which ferment sucrose. This marked correlation between sucrose and raffinose fermentation was emphasized by Howe, who observed that dextrose, lactose, sucrose, and raffinose constitute a metabolic gradient, noting that fermentation of any of these carbohydrates was always accompanied by fermentation of the less complex sugar in the series.

TABLE IV. CORRELATION OF SUCROSE AND RAFFINOSE FERMENTATION IN COLON GROUP.

Acid and gas in raffinose	Acid and gas in sucrose	
	+	—
+	233	8
—	8	84

In a study of 333 strains obtained from soil, sewage, and feces of various animals and man, Levine found only 8 sucrose nonfermenters among 241 strains which attacked raffinose, whereas of 92 raffinose nonfermenters, 84 failed to ferment sucrose.

Both sucrose and raffinose need hardly be employed simultaneously in a study of the colon group. The correlation between fermentability of these carbohydrates has also been observed by Winslow and Walker; Birk; Rogers, Clark and Davis; Kligler; Murray; Rogers, Clark and Lubs; and others. The fact that these two sugars are similar in chemical construction (neither possesses a reacting aldehyde group), may explain the similarity in the behavior of colon bacilli towards them.

The polysaccharids are fermented by relatively few of the species or varieties in the colon group. Ford pointed out that the *Bact. aerogenes* was a starch fermenter and that a few strains also fermented inulin. Glycogen is very rarely attacked. Laybourn reports that *Bact. aerogenes* usually attacks starches from many different sources.

The alcohols have been very frequenlty utilized in investigational studies. Thus dulcitol was employed by MacConkey and Jackson in their classifications, but it is now being generally supplanted by other sugars. Mannitol is fermented by practically all members of the colon group except a small group observed by Rogers in his grain series. The alcohol adonitol was recently suggested as a means of differentiating the fecal from the non-fecal *Bact. aerongenes* and this was adopted by the Committee of Standard Methods of Water Analysis in the 1917 report.

Glycerol has been found by Kligler and later by Levine to correlate well with gelatin liquefaction and they both distinguish *Bact. aerogenes* from *Bact. cloacae* on the basis of fermentation of this material. The alcohols are thus an important group of carbohydrates for studies of the colon group of bacteria.

The glucoside salicin has been recently suggested to supplant dulcitol for classification purposes by Kligler and by Levine and Castellani and Chalmers employ it for primary subdivision of their sucrose negative strains. Salicin fermentation is an important differential test.

Voges Proskauer reaction. (Acetyl methyl carbinol test). By the Voges Proskauer reaction is meant the production of an eosin-like coloration in dextrose broth cultures by some members of the colon group, if made strongly alkaline with potassium hydroxide. It takes its name from the fact that it was first observed by Voges and Proskauer in 1898. The coloration develops slowly from the surface of the medium gradually extending throughout the culture. The test is ordinarily carried out by adding two or three c. c. of 10 percent potassium hydroxide to an equal volume of a 48 to 96 hour dextrose broth culture and after thoro shaking the mixture is allowed to stand exposed to the air. The characteristic eosin-like color will develop in a few hours but it is well to record after 24 hours exposure if negative in a shorter time.

It is suggested that the term Voges Proskauer reaction be restricted to designate the formation of acetyl methyl carbinol from glucose but when referring to its production from other carbohydrates or alcohols, the term **acetyl-methyl-carbinol test** be applied. The nature of the substance being tested for is thus indicated just as is the case with **indol.** The Voges Proskauer reaction has been found very valuable and many investigators have observed that it is characteristic of the colon-like organisms of the soil and grains while it is very rare to encounter intestinal members of the colon group which give this test.

Methyl Red Test. Clark and Lubs in 1915 devised the socalled methyl red test which serves to split the colon group into a methyl-red-positive subgroup, found to be characteristic of the organisms obtained from cow feces and other intestinal sources, and the methyl-red-negative subgroup, which is the predominating type in the soil and on grains.

The test is made by adding a few drops of methyl red indicator to a 0.5 percent dextrose-peptone-(Witte)-dipotassium phosphate culture and noting the reaction; a yellow coloration indicates alkalinity or a negative test and a red coloration denotes acidity or a positive test. The reaction

correlates very well with the Voges Proskauer test and will be considered more in detail in the following pages.

Uric Acid Test. Koser, in studying the utilization of nitrogen from various sources, observed that some members of the colon group (*Bact. aerogenes*) can utilize nitrogen from uric acid, whereas others (*Bact. coli*) can not. He also showed this characteristic to be correlated with the methyl red and Voges Proskauer reaction.

Resume. The colon group obviously includes many varieties and species of bacteria. The monosaccharids and the disaccharid lactose are always fermented with acid and gas formation; nitrates are reduced and milk is acidified and coagulated; but with respect to other tests there is considerable variation within the group.

TABLE V. CHARACTERISTICS OF 333 STRAINS OF COLON GROUP FROM SOIL, FECES AND SEWAGE.

SOURCE	Soil		Feces and sewage		All strains	
Number Studied	177		156		333	
Character	No. pos.	% pos.	No. pos.	% pos.	No. pos.	% pos.
Voges Proskauer Test	142	80.3	9	5.8	151	45.4
Motility	123	69.6	96	61.5	219	65.8
Gelatin	83	46.8	0	0.0	83	29.9
Indol	66	37.3	142	91.1	208	62.4
Sucrose*	165	93.3	76	48.7	241	72.5
Raffinose*	162	91.6	79	50.7	241	72.5
Dulcitol*	74	41.8	68	43.6	142	42.7
Glycerol*	78	43.1	118	76.2	196	58.8
Salicin*	159	89.9	102	66.1	261	78.4
Dextrin*	82	46.4	8	5.1	90	27.0
Inulin*	21	11.9	0	0.0	21	6.3
Starch*	57	32.2	7	4.5	64	19.2

*Acid and gas formation observed.

In general the reactions of the organisms isolated from the soil are quite different from those obtained from various animal feces (horse, sheep, pig, cow, and man) and sewage. In Table V. and figure 1 are shown the frequency of the various reactions.

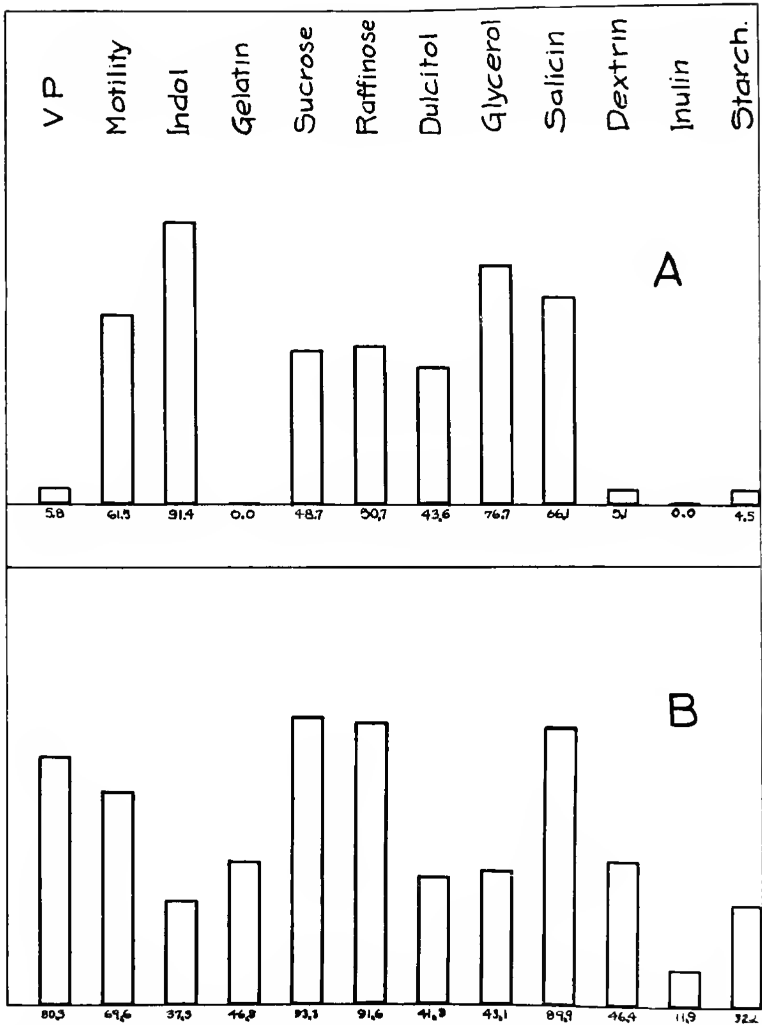


Fig. 1. Percent of Positive Reactions of Coli-like Bacteria from (A) Feces and Sewage, and (B) Soil

II. EVIDENCE OF TWO SUBDIVISIONS IN THE COLON GROUP AND TESTS FOR THEIR DIFFERENTIATION.

The mass of recent work clearly demonstrates that the colon group includes two quite distinct subgroups which differ culturally but particularly with respect to carbohydrate and nitrogen metabolism. These subdivisions, which will be referred to hereafter as the coli and aerogenes sections, are also quite strikingly correlated with habitat, the former predominating in feces and sewage, the latter in the soil and on grains. The evidence for this subdivision together with a detailed consideration of the differential reactions employed is presented here.

General Differential Characteristics. Escherich, in 1885, distinguished *Bact. coli commune* from *Bact.* (*lactis*) *aerogenes* by the greater plumpness of the latter, its lack of motility, and its more rapid coagulation of milk. Later Smith, in 1893 and 1895, indicated that the *Bact. aerogenes* produced a heavier growth and showed a tendency toward capsule formation. He also remarked that gas production was more rapid from glucose and that the proportion of carbon dioxide to hydrogen was greater. Chen and Rettger observe that in glucose broth the volume of gas is seldom greater than 40 percent with the coli section, whereas the aerogense subgroup frequently produced much more gas.

TABLE VI. GAS PRODUCTION IN 1% GLUCOSE BROTH

(After Chen and Rettger, 1920)

	Number of strains	Less than 40%		40% or more	
		No.	%	No.	%
Coli Subgroup	173	165	95.4	8	4.6
Aerogenes Subgroup	447	51	11.4	396	88.6

Burton and Rettger report also that in a modified Uschinsky medium (glucose substituted for glycerol) the coli subgroup grows very poorly, if at all, whereas the aerogenes strains show vigorous growth with almost complete utilization of the sugar.

Temperature Relationships. In some very careful studies on the rate of multiplication of *Bact. coli,* Barber found that the rate increased with increase in temperature showing a maximum at 44 degrees to 45 degrees C. A temperature of 40 degrees C. has often been recommended for the isolation of the colon group from water and in the Eijkman test 46 degrees C. is employed.

With reference to the aerogenes section, we find that Rogers and his associates often mention the necessity for using a relatively low temperature (30 degrees C.) for growth of some strains isolated from grains. This observation is concurred in by Winslow and Cohen, and more recently Chen and Rettger report that in studies of soil organism a large number

refused to grow at 37 degrees C., at least for a while. In some unpublished work the writer has observed that in peptone lactose media at 43 degrees C. (in a water bath) all coli culture studied (16) grew luxuriantly, as evidenced by strong turbidity in 24 hours, but 69 percent of these strains showed no gas or only a bubble in this time. Of 20 aerogenes cultures only 2 showed luxuriant growth, 2 a slight growth, while 16 did not grow at all.

That these two subgroups should show this temperature relationship might be anticipated from a consideration of their respective habitats. The coli section, being most frequently encountered in the animal intestinal tract would naturally have a higher optimum growth temperature than the aerogenes section, which is more characteristic of non-fecal origin. It would be interesting to know whether the members of the aerogenes section, isolated from the intestinal contents of man can be differentiated from the soil and grain strains on the basis of temperature relationship.

EVIDENCE FROM CARBOHYDRATE METABOLISM

The metabolism of carbohydrates, particularly glucose, has been carefully studied by a number of investigators. The work of Harden and Walpole on the products of fermentation of glucose; that of Keys and Gillespie, and of Rogers and his associates on the gas ratio; the observations on the Voges Proskauer test and on acid production particularly the recent studies on the limiting H^+ion concentration have served to clarify the entire group.

The Products of Fermentation of Glucose. Harden has probably done the most important and extensive work on this problem. Among the products of glucose fermentation, Harden and Walpole list alcohol, acetic acid, lactic acid, succinic acid, formic acid, carbon dioxide, and hydrogen as indicated in the following table:

TABLE VII. PRODUCTS OF DECOMPOSITION OF GLUCOSE
(After Harden and Walpole 1905-06)

Products of fermentation	Per cent by weight of sugar fermented by	
	Bact. ærogenes	Bact. coli
Alcohol	17.1	12.85
Acetic acid	5.1	18.84
Lactic acid	5.5	31.90
Succinic acid	2.4	5.20
Formic acid	1.0	0.
Carbon dioxide	38.0	18.1
Carbon dioxide c. c. per gram glucose	198.3	91.8
Hydrogen c. c. per gram glucose	82.4	110.0
CO_2/H_2	2.4	.83
H_2/CO_2	.42	1.19

A perusal of the table will show that *Bact. coli* produces a large amount of acid whereas the acidity produced by *Bact. aerogenes* is very much less. This is particularly noticeable with respect to acetic and lactic acid. It will also be noted that the *Bact. aerogenes* produces about twice as much carbon dioxide as *Bact. coli* and that the volume of hydrogen gas formed is more nearly the same. They observed further that *Bact. coli* utilized only a part of the available carbohydrate whereas the *Bact. aerogenes* strains completely exhausted the sugar. They conclude from these observations that the two organisms act upon glucose in a totally different manner and must therefore be regarded as separate and distinct.

The Voges Proskauer Reaction. If the products of glucose decomposition enumerated above are summed up, it will be found that only 69 percent of the carbon is accounted for in case of *Bact. aerogenes* and 87 percent with *Bact. coli.* This led Harden to search for the discrepancy which he accounted for by the presence of a crude glycol. This consists for the most part of 2:3 butyleneglycol ($CH_3CHOH\text{-}CHOH\text{-}CH_3$), On oxidation it yields acetyl-methyl-carbinol (CH_3, CHOH, CO. CH_3), a volatile reducing substance, which, when mixed with potassium hydroxide in the presence of peptone, imparts an eosine-like coloration to the mixture on standing. Butyleneglycol is oxidized to acetyl-methyl-carbinol by *Bact. aerogenes* but not by *Bact. coli.*

Neither acetyl-methyl-carbinol nor butyleneglycol give the eosin-like coloration when mixed with potassium hydroxide. In the presence of peptone, however, the coloration develops on standing in the case of the carbinol but not with the glycol. According to Harden the reaction is due to further oxidation of the carbinol ($CH_3CO.CHOH.CH_3$) to diacetyl ($CH_3CO.CO.CH_3$) which reacts with some constituent of the peptone. In a later study Harden and Norris report that in the presence of strong potassium hydroxide solution diacetyl reacts with proteins to give a pink coloration together with a green fluorescence. With arginine, creatine, dicyanamide and guanidine acetic acid, the pink coloration is also obtained but the fluorescence is absent. The reaction depends on the presence of the group NH:C (NH_2) N:HR. The exact significance of R. has not been determined. Harden ascribed the Voges-Proskauer reaction to the production of acetyl methyl carbinol.

The reaction takes its name from the fact that it was first observed by Voges and Proskauer in 1898, in their studies on the "Bacteria of Hæmorrhagic Septicæmia." They describe this observation as follows:

"On addition of caustic potash, we observed a new interesting color reaction. If the tube be allowed to stand 24 hours and longer at room temperature, after the addition of the potash, a beautiful fluorescent color somewhat similar to that of a dilute alcoholic solution of eosin forms in the culture fluid particularly at the open end of the tube exposed to the air. We have investigated a few of the properties of this coloring substance, which is not produced by the action of the alkali on the sugar, and have found that it is fairly resistant to the action of the external air. After a

time however, it becomes paler, and finally gives place to a dirty greenish brown."

Considerable work has been carried out on the Voges Proskauer test in the last five years particularly with reference to its constancy, reliability, and methods of determination.

It was customary to allow the potassium hydroxide-culture-mixture to stand 24 hours. and some investigators did not record until after 48 hours. This is extremely unfortunate as it results in unnecessary loss of time. Levine, Weldin, and Johnson found that of 140 strains which gave the Voges Proskauer reaction from glucose, 130 (92.9%) were positive after 5 hours. A similar result was observed with sucrose, where of 134 positive carbinol tests, 127 (94%) were obtained in 5 hours. The same was true of other substances from which acetyl-methyl-carbinol was produced as is shown in the accompanying table. They conclude from this that a period of 5 hours after the addition of the alkali is sufficient as a presumptive test for the Voges Proskauer reaction.

TABLE VIII. COMPARISON OF FIVE HOUR AND TWENTY-FOUR HOUR RECORDS OF TESTS FOR ACETYL-METHYL-CARBINOL.

Test Substances	Total positive reactions	Positive in 5 Hr.		Positive in 24 Hr. Negative in 5 Hr.	
		No.	%	No.	%
Glucose	140	130	92.9	10	7.1
Sucrose	134	127	94.8	7	5.2
Raffinose	114	114	100.0	0	0.0
Mannitol	131	116	88.5	15	11.5
Salicin	104	86	82.7	18	12.3
Dulcitol	14	11	78.6	3	21.4
Dextrin	12	12	100.0	0	0.0
Starch	18	17	94.4	1	5.6

West suggested that the reaction could be hastened by heating the mixture and blowing air through it. Levine, Weldin and Johnson employed various oxidizing agents (potassium dichromate, potassium perchlorate, bleaching powder, barium peroxide, and hydrogen peroxide) all of which were capable of accelerating the reaction but best results were obtained with hydrogen peroxide. To 3 c. c. of a 48 hour culture was added an equal volume of 10 percent potassium hydroxide; the mixture was heated in a boiling water bath for 3 minutes and then 2 or 3 drops of hydrogen peroxide were added. The pink coloration appeared in one or two minutes and persisted for several hours. An excess of hydrogen peroxide or any other oxidizing agent is to be avoided as the coloration will disappear in a very few minutes, or even instantly, if the excess is very great.

Chen and Rettger suggest the following technique for the Voges Proskauer test: Five or six c. c. of the culture are added to an equal volume of 10 percent potassium hydroxide in a test tube, well shaken, and incubated at 30 degrees C. for one to three hours, after which the tube is again vigorously shaken until the liquid becomes foamy. A decided eosin-like coloration will develop in an hour or two.

The test for acetyl-methyl-carbinol is but little affected by the period or temperature of incubation of the culture. Positive reactions have been obtained after one, three, or five days at 30 to 37 degrees C. and Chen and Rettger obtained positive results in 10 to 14 hours at 30 degrees C. The kind of peptone does not influence the test, but brighter reactions are obtained in peptone than in synthetic media. As neither the character of the medium nor the period of incubation of culture interferes seriously with the test, the Voges Proskauer reaction should serve as a convenient and valuable index for the differentiation of aerogenes from the more objectionable coli section.

Gas Production and Gas Ratio. When the presumptive test for the colon group was first suggested, it was pointed out that a volume of 25 to 75 percent gas was particularly likely to be due to colon bacilli. Escherich in 1885 determined gas ratios for *Bact. aerogenes*, but Theobold Smith in 1895 first called attention to the significance of the ratio of the gases evolved in the decomposition of glucose, pointing out that, whereas *Bact. coli* produced twice as much hydrogen as carbon dioxide, the *Bact. aerogenes* differed in that it produced equal volumes of these two gases. These ratios were obtained with the Smith fermentation tube and may be referred to as the crude gas ratio. The determination of the composition of the gases in the Smith tube is very inaccurate and unreliable due in part to the absorption and solution of carbon dioxide and to neutralization by amphoteric substances in the culture medium which would tend to reduce the amount of carbon dioxide observed. Referring to Table VII., it will be noted that in the careful quantitative studies of glucose fermentation by Harden and Walpole, *Bact. coli* evolved carbon dioxide and hydrogen in approximately equal volumes and not in the ratio of one to two as had been observed by Smith. On the other hand, *Bact. aerogenes* forms twice as much carbon dioxide as hydrogen instead of equal volumes observed with the Smith tube. These differences are easily accounted for, as has been stated above, by the loss of carbon dioxide in the Smith tube.

Keyes and Gillespie carried out a series of very careful experiments on the gas ratio and their work has since been confirmed and amplified by Rogers and his associates. They conclude that the accurately determined gas ratio, obtained by growing the test organism in glucose medium in a vacuum and measuring all gas formed, is of fundamental significance and importance in studies on the colon group.

The real significance of this accurately determined gas ratio was not fully appreciated until 1914 when Rogers called attention to the striking correlation between this ratio and the source of the organisms. In three papers by Rogers, Clark, and Davis (1914) and Rogers, Clark, and Evans (1914 and 1915), it is demonstrated very conclusively that the colon strains obtained from bovine feces decompose glucose with the liberation of carbon dioxide and hydrogen in about equal volume, while strains isolated from grains formed two or more times as much carbon dioxide as hydrogen. The former group ($CO_2/H_2=1:1$) seemed in their other characteristics to resemble *Bact. coli;* the latter ($CO_2/H_2=2:1$)

appeared to be the *Bact. aerogenes.* This work of Rogers and his associates aroused considerable interest in the possible different sanitary significance of these bacteria and has stimulated considerable investigational studies.

Acid Production. Studies on acid production have been carried out from two points of view. The carlier observations were restricted to qualitative tests or to the determination of total titratable acid with phenolphthalein as an indicator but the more recent studies have concerned themselves with actual or effective acidity (i. e. the H^+ion concentration); Evidence as to the differentiations of *Bact. coli* and *Bact. aerogenes* by both of these methods will be considered briefly.

Total or Titratable Acid. In considering total acidity, we are struck with the fact that the titer is affected by the composition of the medium as was indicated in the preceding chapter. Observations of different investigators are therefore extremely difficult to compare as comparable data can be obtained only with the same medium. Furthermore in order to avoid fallacies, it is necessary to observe considerable numbers of strains and to treat the results statistically. A few extremely high or low results will influence considerably the average acid production of a collection of organisms. The use of unqualified averages may therefore lead to a misconception of the acid producing properties of a group. To supplement the arithmetic mean or numerical average some statement should be made as to the distribution of the individual strains (variates) about the average. This may be indicated by the probable error or by the standard deviation. The cœfficient of variability (the ratio of the standard deviation to the mean) is an excellent abstract measure of variability. The modal acid production (the amount of acid most frequently formed) is usually of greater significance than the average amount of acid formed.

The standard deviation is the measure of variability most commonly employed, particularly by mathematicians. It may be expressed mathematically as

$$\theta = \sqrt{\frac{\Sigma d^2 f}{n}}$$

where "n" is the number of variates or observations, "d" the deviation of the individual variates from the mean, and "f" the frequency of a deviation "d".The standard deviation serves to indicate whether the departures from the mean are small or great. The closer the individual organisms group themselves about the mean, or average, the smaller the standard deviation.

An example may make clear the meaning and significance of the standard deviation. Suppose that the amounts of acid formed by a group (A) of 4 organisms in glucose broth are 2.1, 2.2, 2.2, and 2.3 percent normal acid, and that those formed by another group (B) of 4 organisms are 1.9, 2, 2.4, and 2.5 percent normal acid. The average for each group is 2.2, but mere inspection shows that the organisms in Group A and those in

Group B are quite differently distributed with respect to this average. In large collection of data inspection is impracticable but the standard deviation serves well in its place. The standard deviation in Group A is ±0.07 while for Group B it is ±0.25. The larger deviation in B denotes that the individuals in the group wander farther away from the average than do those in Group A.

Acid Production from Glucose. Considering 156 strains, isolated from various sources (including sheep, horse, cow, pig, man, and sewage), the V. P. negative (coli section) gave an average of 1.82 percent normal acid with an empirical mode at 1.9 percent while the V. P. positive (aerogenes section) produced an average of 1.46 percent with a mode at 1.5 percent normal acid. Although the difference (0.36±0.04) is not very large, it appears significant for rather striking differences in acid formation between the V. P. positive and V. P. negative strains are observed with many other test substances, as maltose, sucrose, glycerol, and dulcitol. This observation is also in line with the work of Harden and Walpole, already referred to that *Bact. aerogenes* produces less acid than *Bact. coli* from glucose.

TABLE IX. ACID PRODUCTION BY COLON GROUP FROM GLUCOSE AND SUCROSE.

Percentage of normal acid	Glucose			Sucrose*		
	All strains	Voges-Proskauer		All strains	Voges-Proskauer	
		Negative	Positive		Negative	Positive
0.00-0.19				79	79	
0.20-0.39				1	1	
0.40-0.59				8	8	
0.60-0.79	1	1		48	48	
0.80-0.99	3	3		6	6	
1.00-1.19	2	1	1			
1.20-1.39	11	9	2	3	1	2
1.40-1.59	19	15	4	7	3	4
1.60-1.79	22	20	2	2		2
1.80-1.99	54	54				
2.00-2.19	41	41		1		1
2.20-2.39	3	3				
Total acid-formers	156	147	9	75	66	9
Mode	1.90	1.90	1.50	0.70	0.70	1.50
Mean	1.80	1.82	1.46	0.84	0.74	1.57
Probable error	±.20	±.20	±.12	±.23	±.14	±.16
Standard deviation	±.30	±.30	±.18	±.34	±.20	±.23

*Only fermenters included in calculations.

Acid Production from Sucrose. Sucrose is not attacked by all members of the colon group. It is practically always fermented, however, with acid and gas production by the aerogenes section and many of the strains of the coli section. In the foregoing table are given the fre-

quency of acid production from sucrose by the V. P. negative (coli) and V. P. positive (aerogenes) strains isolated from feces and sewage. It will be observed that those V. P. negative forms which are capable of

TABLE X. ACID-PRODUCTION IN FERMENTABLE SUBSTANCES BY VOGES-PROSKAUER-POSITIVE AND NEGATIVE BACILLI OF COLON GROUP

Test Substance	Percent of Normal Acid				Excess of acid (in percent of Normal) by the V.P.+ strains	
	American-Museum strains		Levine's strains			
	V.P.—	V.P.+	V.P.—	V.P.+	American-Museum strains	Levine's strains
Glucose	1.82	1.52	1.82	1.46	—.30	—.36
Galactose	1.31	1.28	1.36	1.21	—.03	—.15
Lactose	0.96	0.85	1.31	1.26	—.11	—.05
Mannitol	1.37	1.41	1.31	1.48	+.04	+.17
Maltose	0.66	1.01	0.75	1.12	+.35	+.37
Sucrose	0.71	1.52	0.74	1.57	+.81	+.83
Raffinose	0.79	1.22	0.96	1.32	+.43	+.36
Glycerol	0.58	1.27	0.70	1.28	+.69	+.58
Dulcitol	0.83	1.15	0.81	1.30	+.32	+.49
Salicin	1.00	1.38	0.94	1.28	+.38	+.34

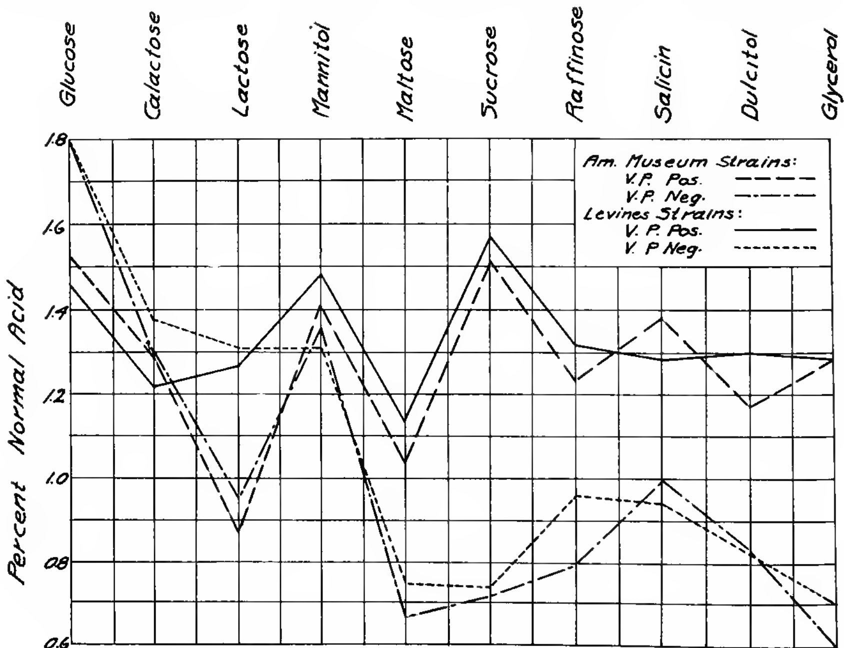


Fig. 2 Acid-Production by Voges-Proskauer Positive and Negative Coli-like Bacteria

attacking sucrose produce less acid than the V. P. positives. The means for the two groups are .74 and 1.57 percent respectively and the empirical modes 0.7 and 1.5 percent normal acid.

Acid Production from Various Other Carbohydrates. Observations on the average quantities of acid formed from various substances by members of the coli (V. P.—) and aerogenes (V. P.+) subgroups, obtained from the American Museum collection and those isolated by the author from feces and sewage gave the results shown in Table X.

Inspection of Table X. and figure 2 indicates that considering all of the 167 strains studied, the aerogenes strains form less acid from glucose than does the coli section and about equal quantities form galactose, mannitol, and lactose. In all other test substances, maltose, salicin, raffinose, dulcitol, glycerol, and sucrose, the V. P. positive strains (aerogenes) give rise to considerably more acid, the excess increasing in the order named. Although the differences obtained in salicin, raffinose, and possibly glucose, may not be so significant on account of the variation observed among individual strains, it is nevertheless quite striking that a small number of strains taken at random, from the American Museum collection and a few freshly isolated cultures should show such a marked parallelism as is indicated in Table X. and figure 2 with respect to the amount of acid formed when they decompose such a variety of carbohydrates.

The H^+ion Concentration. In 1915 Clark and Lubs first pointed out that in a medium consisting of 0.5 percent anhydrous glucose, dipotassium phosphate, and Witte's peptone, the low ratio cultures (coli section) produced a high acidity (H^+ion) which remained permament; the high ratio strains (aerogenes section) were much less acid and became progressively more alkaline. This difference in acidity was easily recognized by the use of the indicator methyl red which gave an acid reaction with the low ratio and an alkaline reaction with the high ratio group. This test has become known as the **methyl red reaction.**

Principle of the Methyl Red Reaction. Michælis and Morcora* observed that cultures of *Bact. coli* fermented lactose until a hydrogen ion concentration of 1×10^5 was reached and then ceased their activity. They considered this point a physiological constant for the organism in question. Clark, in 1915, obtained similar results with a culture of *Bact. coli* in glucose peptone water. He found that, irrespective of the initial acidity, the final hydrogen ion concentration varied but slightly (PH 4.37 to 4.55). If we again consider the products of decomposition of glucose by *Bact. coli* and *Bact. aerogenes* (see Table VII.), it will be noted that whereas *Bact. aerogenes* decomposes 14 percent of glucose into acids (acetic lactic, succinic, and formic) *Bact. coli* produces acids from 56 percent of the glucose. Thus, from a given quantity of sugar, the amount of acid evolved and consequently the H+ion reached will be greater for *Bact. coli* than for *Bact. aerogenes.* If now the amount of glucose in a medium is restricted to that quantity necessary to yield the limiting (inhibiting) H^+ion concentration for *Bact. coli,* the resulting reaction with *Bact. aerogenes* will

*Cited by Clark and Lubs

necessarily be less acid. On further incubation the *Bact. coli* would die off, whereas the more alkaline *Bact. aerogenes* culture would continue to grow exhausting the available sugar, after which the reaction would become progressively more alkaline. This reversion is due partly to the decomposition of the peptones with the liberation of alkali but particularly to the conversion of the organic acids originally produced from the glucose into carbonates and bicarbonates, as shown by Ayers and Roup. As incubation is prolonged, the difference in reaction between a *Bact. aerogenes* culture and one of *Bact. coli* may be considerably increased.

Clark and Lubs, in some very careful work, found that a concentration of 0.5 percent anhydrous glucose, dipotassium phosphate, and Witte's peptone affords the proper combination of glucose and buffer substances for the differentiation of the coli and aerogenes sections. They recommend an incubation temperature of 30 degrees C. for 5 days, after which period, *Bact. coli* is acid and *Bact. aerogenes* will be found to be alkaline to the indicator methyl red.

TABLE XI. EFFECT OF THE TEMPERATURE AND THE PERIOD OF INCUBATION ON THE REACTION WITH METHYL RED.

Reaction	Incubation at 37 C. in days				Incubation at 30 C. in days			
	2nd	3rd	4th	6th	2nd	3rd	5th	7th
Acid	143	146	146	146	139	149	148	148
Neutral	12	9	9	9	18	6	7	6
Alkaline	12	12	12	12	10	12	12	13

As to the effect of temperature and period of incubation on the reaction with methyl red, the indications are that the final reaction is reached more quickly at body temperature than at 30 degrees C. (Table XI.). With an incubation period of 5 days there is little choice between 30 degrees C. and 37 degrees C., but if the time of incubation is reduced to two or three days, which would he very desirable for routine water work, the body temperature seems preferable. It is of course recognized that some strains of the aerogenes section will not grow well at body temperature, but from the point of view of the bacterial analysis of water it is felt that there is little value in detecting such strains. The extra expense of maintaining a 30 degree incubator for their isolation is consequently not warranted.

Too much emphasis can not be placed on the necessity of employing Witte's peptone in preparation of the medium mentioned above. The indiscriminate substitution of other peptones is bound to lead to confusion and error. Owing to the great difficulty in obtaining Witte's peptone, Clark and Lubs were led to devise a synthetic medium which consists of the following:

0.7% anhydrous Na_2HPO_4.
0.2% acid potassium phthalate.
0.1% aspartic acid.
0.4% anhydrous dextrose.

Incubation at 30 degrees C. for three to five days is recommended. This synthetic medium may also be used for the Voges Proskauer test by mixing with it (at the time of addition of the alkali) 0.1 percent pure casein.

Evidence from Nitrogen Metabolism. In the foregoing paragraphs it was pointed out that there were two main divisions in the colon group which could be easily distinguished by differences in carbohydrate metabolism. It is becoming apparent that the differentiation may also be made upon nitrogen metabolism.

In 1903, Rettger noted that the products of protein metabolism (mercaptan, scatol, phenols aromatic axyacids, etc.) are formed much more slowly by *Bact. aerogenes* than by *Bact. coli.*

Koser (1918) in a study of 124 colon cultures found that in a medium containing uric acid as the sole source of nitrogen the V. P.+ strains (aerogenes section) grew luxuriantly whereas the V. P.— strains (coli section) failed to grow.

TABLE XII. DIFFERENTIATION OF COLI AND AEROGENES SECTIONS
(After Koser, 1918)

	Growth in Uric Acid medium		Voges Proskauer reaction		Methyl Red Test	
Cultures employed	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Coli subgroup 74	0	74	0	74	72	2
Aerogenes subgroup 50	50	0	50	0	0	50

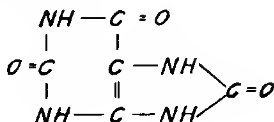
The medium employed by Koser consisted of the following:

Distilled ammonia free water	1000 c. c.
NaCl	5 grams.
Magnesium sulphate	0.2 grams.
$CaCl_2$	0.1 grams.
Dipotassium phosphate	1.0 grams.
Clycerol	30.0 grams.
Uric acid	0.5 grams.

Sterilization is in the autoclave at 13 to 15 pounds for 15 minutes. A slight turbidity, presumably due to the finely divided precipitate of calcium sulphate may be evident immediately after autoclaving but disappears on cooling.

A solid uric acid medium may be prepared by the addition of 1.5 percent washed agar.

He suggests the differentiation is due to the inability of the strains in the *Coli Section* to break down the purin ring and thus obtain nitrogen for growth from the uric acid.



Evidently scrupulous care must be employed in the preparation of the medium to avoid introduction of any nitrogenous compounds which might be present in imperfectly cleaned glass-ware or ordinary tap water or even distilled water, for the nitrogen thus introduced might serve to promote growth of organisms of the coli subgroup and thus obscure the differential reaction.

The reliability of this uric acid differential test has been confirmed by Chen and Rettger in a study of 640 strains isolated from soil and from intestinal tracts of man and various animals. They note, however, that half the coli strains isolated from the soil grew in the uric acid medium.

TABLE XIII. GROWTH OF THE COLON GROUP IN URIC ACID MEDIUM

(After Chen and Rettger, 1920)

Growth	Pos.	Neg.	Total
Aerogenes strains from soil (V. P. +)	447	0	447
Coli strains from feces (V. P. —)	0	173	173
Coli strains from soil (V. P. —)	10	10	20

They showed further that xanthine may be substituted for uric acid and that all the coli strains from the soil failed to grow in the xanthine medium. It is probable that xanthine will be found preferable to uric acid for differentiation of the coli from the aerogenes types but there may be an intermediate group resembling the former with respect to the V. P. and methyl red reactions but which differs from it in its ability to attack uric acid.

The temperature of incubation employed by Koser was 37 degrees C. for four days. Chen and Rettger employed a temperature of 30 degrees C. for three to five days.

Differentiation on Solid Media. The coli and aerogenes sections also present several cultural differences particularly with respect to the appearance of colonies on some solid media. The aerogenes colonies are generally larger more opaque and more convex than those of the coli subgroup. On litmus lactose agar the former sometimes revert to an alkaline reaction. These differences, however, are very difficult to detect on litmus lactose agar but may be readily observed in the Endo and particularly on eosin-methylene-blue agar.

Ferreira, Horta and Paredes noted that *Bact. aerogenes* and *Bact. cloacae* produced a rose color on Endo agar but that the metallic luster, so characteristic of *Bact. coli*, was absent. Levine employing a simplified fuchsin sulphite (Endo) agar notes striking differences between the colonies of the coli and aerogenes subgroups. The coli strains formed deep red button-like colonies with a greenish metallic sheen (by reflected light), and were usually three or four m. m. in diameter when well isolated. The *aerogenes* colonies are lighter colored, markedly convex, and do not show the metallic luster.

Wood records that on neutral-red-bile salt lactose agar, the V. P.+M. R.— strains may develop mucoid colonies which are generally paler than the V. P.—M. R.+ strains.

Another distinct differentiation may be obtained with the modification of the (Holt-Harris) eosin-methylene-blue agar (see appendix). On this medium the well isolated coli colonies are about three m. m. in diameter appearing very dark, almost black by transmitted light and by reflected light they seem to be button-like, often concentrically ringed with a distinct greenish metallic sheen. Those of aerogenes, on the other hand, are larger, tend to run together, are markedly convex, very much lighter in color, (when viewed by transmitted light), and a metallic sheen is rarely observed. That these cultural differences are quite reliable, at least for routine work, is indicated by a report of Levine, who found that 96.9 percent of 122 colonies picked as of the coli section from their appearance on eosin methylene blue agar and 82.4 percent of 102 colonies supposedly of the aerogenes section were confirmed by subsequent tests.

Correlation of Reactions. The studies of carbohydrate and nitrogen metabolism and cultural characters, all indicate that the colon group embraces two distinct sections which may be readily distinguished by a number of tests including (1) the gas ratio, (2) acidity to methyl red, (3) the Voges Proskauer reaction, (4) growth in uric acid medium, and (5) appearance of colonies on agar (Endo and eosin-methylene-blue).

Although no investigator has employed all these reactions simultaneously, they are known to be very strikingly correlated. Thus Clark and Lubs in 1915 observed a perfect correlation between the gas ratio and the methyl red test. Levine pointed out that the strains which were positive for methyl red were negative for the V. P. test and vice versa. The correlation between these two reactions had been extensively confirmed. Johnson; Burton and Rettger; Chen and Rettger; Hulton; Greenfield; and many others have observed an almost perfect correlation. Clark and Lubs (1917) correlated the gas ratio with the V. P. and M. R. reactions, while Koser and Chen and Rettger have shown that the uric acid test, the V. P. and methyl red reaction were strikingly confirmatory of each other.

Choice of a Routine Differential Test. The question naturally arises as to which test shall be employed or at least given preference in future studies of this group. A choice of a test must of course be dependent upon the nature of the work at hand. For investigational studies it should be emphasized that the carefully determined gas ratio, as is urged by Rogers, Clark, etc., is of fundamental importance and that in order to throw further light upon the reliability of the other reactions mentioned it is desirable that they should all be considered and observed. The problem, however, is quite different when applied to routine water analysis. There the time available is limited, the apparatus and other laboratory facilities are at a minimum, and the skill of the analyst, we must regrettably admit, is too frequently not comparable with that of the chemist.

For routine water work the selection of a differential test must be governed to a great extent by the following considerations:

1. The medium should be simple, easy to prepare, and permit of a considerable degree of variation.

2. The reaction should be distinct and constant.

3. The test must be one which can be completed in a short time, preferably not more than 24 hours.

In the light of these considerations it becomes evident that the gas ratio is out of the question. That it is permanent and constant has been well demonstrated by the investigations of Rogers and his associates, but the skill and apparatus necessary together with the time required removes it from the possibility of a routine water test.

The uric acid reaction is a convenient and reliable test but the scrupulous care required in preparation of the medium can not be obtained at the present time at least, in laboratories which are concerned with routine water analysis. The period of incubation, three to five days, is also a disadvantage.

The choice thus becomes limited to the Voges-Proskauer and methyl red tests. Opinions are quite at variance as to which is preferable. The methyl red reaction has been urged by Clark and Lubs and by Winslow. The necessity for employing Witte's peptone together with the fact that the reaction is based on a delicate adjustment of the source of carbon and buffer substance and the insatiable desire among bacterialogists to deviate from the media recommended and to make individual substitutions has lead to numerous difficulties in the application of this test in practice. The synthetic medium of Clark would of course eliminate some of these difficulties. The methyl red reaction is simple, reliable, and when carefully performed, constant, but the period of incubation for accurate differentiation is quite long (3 to 5 days), too long, in fact, to be conveniently employed as a routine test.

The tendency recently has been toward the V. P. reaction. Thus Chen and Rettger conclude from a study of 640 strains that the V. P. reaction is even more satisfactory than the methyl red test in that it is simple in operation, and when correctly carried out, is thoroughly constant in its results.

Clark and Lubs, on the other hand, point out that the production of acetyl methyl carbonol, being the result of secondary reaction, and possibly synthetic to some extent, may not be intimately connected with the main course of the fermentation and the quantity produced may be very slight, thus giving a faint V. P. test even though the fermentation may be very vigorous. Nevertheless, the reaction has been found to be very constant and it has proven very satisfactory in the hands of practically all who have tried it. The advantages of this reaction are:

1. Any peptone medium in which the organisms will grow and which contains glucose (in a wide range of concentration) is suitable. It is preferable, however, to have the medium as free from color as possible.

2. The reaction may be obtained after 14 to 24 hours incubation at 30 degrees or 37 degrees C.

3. The brand of peptone employed does not affect the intensity of the reaction.

It is interesting to note that in case of a mixture of coli and aerogenes types, the methyl red test will be acid and the V. P. reaction will be positive. Undoubtedly many instances are recorded as examples of intermediate strains when they were really due to impure cultures. This was the experience of Johnson and Levine who found that by repeated purification, the proportion of strains which would not show perfect correlation was considerably reduced. Chen and Rettger record that of 18 strains which persisted in giving methyl red positive and V. P. positive reactions in all media, which they employed, only four continued to give non-correlating reactions after purification, although contaminating organisms could not be demonstrated.

Resume. The gas ratio, Voges Proskauer, methyl red, and uric acid tests are strikingly correlated. The members of the colon group which produce acetyl methyl carbinol, are capable of using the nitrogen from the purin ring of uric acid, give an alkaline reaction with the methyl red test, and in the decomposition of glucose, yield a relatively small quantity of acid and two or more times as much CO_2 as H_2. On the other hand, the organisms, which do not produce acetyl methyl carbinol, can not utilize the nitrogen from the purin ring, give an acid reaction with methyl red, break down glucose with the production of a relatively large amount of acid and liberate CO_2 and H_2 in approximately equal volumes. The colon group therefore includes two distinct subdivisions which are characteristically of different sources. These have been designated the coli and aerogenes sections. Their characteristics are tabulated below:

TABLE XIV. DIFFERENTIATION OF THE MAIN SUBDIVISIONS OF THE COLON GROUP.

Section	Gas Ratio CO_2/H_2	M. R. Test	V. P. Test	Growth Uric Acid medium	Habitat
Coli	1.0 (low ratio)	acid	neg.	Negative (no growth)	Predominates in feces and sewage
Aerogenes	1.5 or more (high ratio)	alk.	pos.	Positive (good growth)	Predominates in soil and on grains

III. CLASSIFICATIONS OF THE COLON GROUP OF BACTERIA.

Several attempts have been made to classify the numerous organisms of the colon group for the most part on the basis of acid and gas production from various carbohydrates. The reliability of such studies is sometimes questioned on the ground that fermentation reactions are inconstant and may be easily acquired or lost.

Smirnow grew colon strains in media containing various chemicals (3.0% glucose, 4.0% sodium chloride, 0.5% sodium sulphate, and 0.25 to 0.75% phenol) and found that after successive transfers, for periods of one to three months, indol formation and later fermentation of carbohydrates were suppressed. Reversion to the original characteristics took place rapidly, however, when grown on ordinary media.

Bronfenbrenner and Davis found colon organisms in food which fermented lactose slowly when first isolated but after cultivation on lactose media the rate of decomposition of this substance became normal.

Twort, Penfold, and others, observed spontaneous mutations such as the loss or acquistion of the ability to ferment various carbohydrates.

After a careful survey of the data on biological variations and mutations, Winslow concludes that "Taking the great mass of colon typhoid strains, as they are isolated from the bodies or intestines of man and animals, and cultivated under standard conditions, fermentative characteristics exhibit a high degree of constancy and what is even more important a higher degree of correlation with other biochemical and serological and pathogenic properties."

From the practical water analysis view point, we are especially interested in the effect of a long sojourn in water or soil on the biological activities of *Bact. coli.*

Houston repeatedly found that the proportion of strains not forming indol was much greater among those isolated from purified than from raw waters. He thought this was due to a loss of indol producing power as a result of unfavorable conditions encountered in water. He speaks of such indol negative forms or strains, which differ from the supposedly original *Bact. coli commune*, as "atypical" *Bact. coli.*

Horrocks in 1903 exposed *Bact. coli* in various types of soils and waters for two to three months, and Savage in 1905 observed the effect of tidal mud. They came to the conclusion that alterations in characteristics were not induced and that there was no evidence that *Bact. coli* ever becomes "atypical."

MacConkey placed a broth culture of *Bact. coli* in a sterile Pasteur candle which was then suspended in tap water. The water was changed occasionally. *Bact. coli* was isolated from the candle at irregular intervals up to 358 days and examined biologically and biochemically. In no instance was a loss or gain of a character detected.

It seems that the remarkable facts are not that an organism may occasionlly show a biological variation, but that, considering the simplicity of the bacterial cell, such variations are so infrequent.

Theobald Smith, Kligler, Winslow and others have reported that with cultures of *Bact. cloacae* motility and fermentation of carbohydrates persisted after the power to liquefy gelatin had disappeared. Digestion of gelatin is generally considered a reliable differential test. We therefore need have no compunction about utilizing the apparently more persistent fermentation reactions in studies on classification of the colon group.

Theobald Smith, 1893, suggested that sucrose fermentation may be employed to subdivide the colon group into two subgroups and in 1901 Durham suggested the name *Bact. coli communior* for the sucrose positive variety and noted that the fermentation of starch was distinctive of *Bact. lactis aerogenes.*

MacConkey's Classification. In 1905 MacConkey divided lactose fermenting (acid and gas) bacilli into four groups on the basis of fermentation of sucrose and dulcitol.

TABLE XV.

MacConkey group	Acid and gas		Type species
	Sucrose	Dulcitol	
I	—	—	*Bact. acidi lactici*
II	—	+	*Bact. coli (commune)*
III	+	+	*Bact. neapolitanum*
IV	+	—	*Bact. (lactis) aerogenes*

In 1909 he recognized in each group a number of varieties which were distinguished on the basis of their reactions to the Voges Proskauer test, motility, indol production, gelatin liquefaction, fermentation of inulin adonitol, and inosite. The probable existence of 128 different strains or 32 varieties in each of his four subdivisions was suggested. Many of these have been isolated, described, and given specific names. Others have been merely indicated by a number in his classification as shown in Table XVI.

Bergey and Deehan's Varieties. Very similar to MacConkey's classification is that of Bergey and Deehan (1908). They employed eight characters—fermentation of sucrose, dulcitol, adonitol, and inulin; gelatin liquefaction, indol production, motility, and the Vogese Proskauer reaction—and from a consideration of all possible combinations recognized the possible existence of 256 varieties.

The Jackson Classification. In 1911, Jackson proposed a classification resembling that of MacConkey but preference is given to dulcitol over sucrose for the primary division. Each of the four groups thus formed, which are regarded as species, are then further divided on raffinose and mannitol into four varieties designated (A, B. C, and D), and further differentiation may then be made on motility, indol, gelatin liquefaction, fermentation of other carbohydrates, etc., giving subvarieties, indicated by numerical suffixes (A_1, A_2, B_2, etc.). This scheme which was included in the Standard Method for a Water Analysis for 1912 is detailed in Table XVII.

TABLE XVI. MACCONKEY'S CLASSIFICATION OF LACTOSE FERMENTING BACILLI.

MacConkey Group	Variety No.	Variety (Name)	Gelatin Liquefaction	Motility	Indol	Adonit	Inulin	Inosit	V. P.
Group I.	1		—	+	+	+	—	—	—
(Nos. 1-32)	2	*B. acidi lacti (Huppe)*	—	—	+	+	—	—	—
Sucrose—	3	*B. levans*	+	+	—	—	+	—	+
Dulcitol—	4	B. Grunthal, *B. sulcatus gasoformans*, *B. castellus*	—	+	+	—	—	—	—
	5	*B. vesiculosus*	—	—	+	—	—	—	—
	6		—	—	+	—	—		+
	7		—	+	—	—	—	—	—
	8		—	—	—	—	—	—	
Group II.	33		—	+	+	+	—	—	—
(Nos. 33-64)	34	*B. coli communis*, *B. cavicida*	—	+	+	—	—	—	—
Sucrose—	35	*B. Schafferi**	—	—	+	—	—	—	—
Dulcitol+	36		+	+	—	—	—	—	—
Group III.	65	*B. oxytocus perniciosus*	+	—	+	+	+	+	+
Nos. 65-96)	66		—	—	+	+	—	—	—
Sucrose+	67		—	—	—	+	—	+	+
Dulcitol+	68	*B. rhinoscleroma*, *B. Friedlander*	—	—	—	+	—	+	—
	69		+	+	—	—	+	—	+
	70		+	+	—	—	+	—	—
	71		—	+	+	—	—	—	—
	72	*B. neapolitanus*	—	—	+	—	—	—	—
	73		+	+	—	—	—	—	+
	74		—	+	—	—	—		—
	75		—	—	—	—	—	+	+
Group IV.	97		+	—	+	+	+	—	+
(Nos. 97-128)	98		—	—	—	+	+	+	+
Sucrose+	99		—	—	—	+	+	+	—
Dulcitol—	100		—	+	+	+	—		—
	101		—	—	+	+	—	+	—
	102		+	+	—	+	—		+
	103	*B. lactis aerogenes*, *B. dysenteriae vitulorum*, *B. capsulatus (Pfeiffer)*	—	—	—	+	—	+	+
	104	*B. gasoformans non-liquefaciens**	—	—	—	+	—	+	±
	105		+	+	—	—	+	—	+
	106		—	+	+	—	—	—	—
	107	B. *coscoroba*	—	—	+	—	—	—	—
	108	*B. cloacae*	+	+	—	—	—	±	+
	109		—	+	—	—	—	+	—

*Usually produce only a small amount of gas.

TABLE XVII. THE JACKSON CLASSIFICATION OF COLON GROUP.

Dextrose+
Lactose+

- Dulcite+
  - Saccharose+ — *B. communior*
  - Saccharose— — *B. communis*
- Dulcite—
  - Saccharose+ — *B. aerogenes*
  - Saccharose— — *B. acidi-lacti*

Varieties:

Dulcite+, Saccharose+ — *B. communior*

Variety		Man.	Raf.
(A)	(1)	+	+
(B)	(2)	“ +	“ +
(B)		“ +	“ —
(C)		“ —	“ +
(D) *		“ —	“ —

Dulcite+, Saccharose— — *B. communis*

Variety	Man.	Raf.
(A)	+	+
(B)	“ +	“ —
(C)	“ —	“ +
(D)	“ —	“ —

Dulcite—, Saccharose+ — *B. aerogenes*

Variety		Man.	Raf.
(A)	(1)	+	+
(A)	(2)	“ +	“ +
(A)	(3)	“ +	“ +
(B)	(1)	“ +	“ +
(A)	(2)	“ +	“ +
(C) *		“ —	“ +
(D) *		“ —	“ —

Dulcite—, Saccharose— — *B. acidi-lacti*

Variety		Man.	Raf.
(A)	(1)	+	+
(A)	(2)	“ +	“ +
(B)		“ +	“ —
(C)		“ —	“ +
(D)		“ —	“ —

+ Positive reaction.
— Negative reaction.
* Unknown variety.
1-2-3 subvarieties.

Castellani and Chalmers' Grouping. These authors have recently suggested a rather unique classification of bacteria in which the organisms generally considered as members of the colon group are distributed among three Tribes and three Genra as follows:

Tribe Encapsulateae. (Castellani and Chalmers, 1918. Bacillaceæ growing well on ordinary media, without endospores; neither fluorescent nor chromogenic; aerobes; facultative anaerobes; not liquefying gelatin; possessing capsules in animal tissue.

The genus *Encapsulatus* includes two lactose fermenting (acid and gas) species differentiated on fermentation of inosite.

Inosite not fermented. 1. *Encapsulatus acidi lactici*
Inosite fermented with acid and gas. 2. *Encapsculatus lactis aerogenes*

Tribe Proteae. (Castellani and Chalmers, 1918) *Bacillaceae* growing well on ordinary media; without endospores; aerobes; neither fluorescent nor chromogenic; aerobes; facultative anaerobes; not liquefying gelatin,

They recognize two lactose fermenting species in the genus *Cloaca* which are different on sucrose fermentation.

Sucrose fermented with acid and gas. 1. *Cloaca cloacae.*
Sucrose not fermented 2. *Cloaca levens.*

All other members of the colon group are placed in their *Genus Escherichia* of the Tribe Ebertheae.

Tribe Ebertheae. (Castellani and Chalmers, 1918). Bacillaceae growing well on ordinary laboratory media; not forming endospores; aerobes, and often facultative anaerobes; without fluorescence pigment formation or gelatin liquefaction; without polar staining; Gram negative; without a capsule.

Genus Escherichia. (Castellani and Chalmers, 1918.) *Ebertheae* which ferment glucose and lactose completely with acid and gas; milk clotted.

I. Indol positive division. (Smith)
  A. Sucrose fermented—**Communior section of Durham.**
    1. Dulcitol fermented with acid and gas.
      a. Adonitol fermented with acid and gas.
        (1) Motile 1. *Escherichia oxytocus*
        (2) Non motile. 2. *Escherichia metacoli*
      b. Adonitol no change.
        (1) Motile.
          (a) Agglutinated by pseudocoli serum
            3. *Escherichia pseudo-coli*
          (b) Not agglutinated (late fermentation of sucrose)
            4. *Escherichia coliformis*
        (2) Non motile. 5. *Escherichia neapolitanus*
    2. Dulcitol not fermented.
      a. Motile
        (1) Inosite fermented with acid and gas.
          6. *Escherichia pseudocoloidella*
        (2) Inosite no change 7. *Escherichia pseudocoloides*

b. Non motile. 8. *Escherichia pseudocoscoroba*

B. Sucrose not fermented—**Communis section of Durham.**

1. Dulcitol fermented with acid and gas.

a. Salicin fermented with acid production.
9. *Escherichia cavicida*

b. Salicin fermented with acid and gas.

(1) Motile. 10. *Escherichia coli*

(2) Non motile.

(a) Inosite not fermented.
11. *Escherichia coloidella*

(b) Inosite fermented with acid and gas.
12. *Escherichia coloides*

c. Salicin no change. 13. *Escherichia metacoloides*

2. Dulcitol—no change.

a. Motile.

(1) Maltose—acid and gas.
14. *Escherichia paragrünthali*

(2) Maltose—no change.
15. *Escherichia grünthali*

b. Non motile.

(1) Maltose—acid and gas.
16. *Escherichia colitropicalis*

(2) Maltose—no change.
17. *Escherichia vesiculosus*

II. Indol negative division (Smith). 18. *Escherichia coli mutabilis*

The prominence given to the indol reaction and the fermentation of dulcitol, adonitol, and maltose by Castellani and Chalmers is quite at variance with the practices of American investigators. From the extensive recent studies on carbohydrate and nitrogen metabolism it appears that the acidi-lactici types are more closely affiliated with the strains in their *Genus Escherichia,* than with the lactis-aerogenes forms and that the *Genus Cloaca* of Castellani and Chalmers should be associated with the lactis-aerogenes forms rather than with the proteus group.

A very serious objection to such classifications as those of MacConkey, Bergey and Deehan, and Jackson is their extreme flexibility and complexity; for, as the number of fermentable substances or other characters observed increases, the number of "varieties" increases geometrically (approaching infinity) and soon produces a most unwieldy scheme. The number of "varieties" is given by the formula 2^n where "n" is the number of characters studied. Thus with eight characters there are 256 possible combinations or "varieties." This number rises to 1,024 with 10 characters and to 65,536 when 16 tests are considered. It is aparent therefore that to regard each character as of similar and equal differential value will quickly result in an unwieldy grouping.

Another objection to these classifications is the arbitrary manner of selecting the order in which reactions are to be employed for division. Organisms which are very closely related may be far separated in two

schemes of classification if the authors happen to select different characters for the initial subdivision.

Statistical Classification of the Colon Group. To offset these objections, Winslow suggested the utilization of the statistical method first employed by Andrews and Horder and Winslow and Winslow in studies on *Coccaceae.* Individual characters are not considered paramount and independently but only in relation to each other.

Howe first attempted the statistical method in 1912. This investigator made a detailed study of acid and gas production and various other tests on 630 strains freshly isolated from human intestinal contents. He concluded that indol, nitrate reduction, motility, fermentation of dulcitol and mannitol, and starch were not correlated with other characters and were consequently not of classificatory value. He recognized only two groups the sucrose positive, *Bact. communior,* and the sucrose negative, *Bact. communis.*

Rogers and his associates in 1914 and 1916 studied a large number of colon strains from milk, grains and bovine feces and on the basis of accurately determined gas ratio from dextrose concluded that two distinct groups may be distinguished. One, referred to as the low ratio group, produced carbon dioxide and hydrogen in equal volumes and includes about 52 percent of the strains from milk, 99.6 percent of the strains from bovine feces, and only 4.8 percent of their grain strains; whereas the high ratio group, which was characterized by the production of two or more times as much carbon dioxide as hydrogen, included 47.5 percent of the milk strains, only 0.5 percent of the bovine fecal strains, and about 95 percent of the strains obtained from grains. There is thus a very strong correlation between these subdivisions and the source.

Kligler (1915) suggested that salicin be substituted for dulcitol, in subdividing coli-like bacteria, pointing out that salicin fermentation correlates better with the Voges-Proskauer reaction than does dulcitol decomposition. He recognizes a sucrose negative-salicin negative group (*B. acidi-lactici*); sucrose negative-salicin positive group (*B. communis*); sucrose positive-salicin negative group (*B. communior*) and sucrose positive-salicin positive (*B. aerogenes*). *B. cloacae* is differentiated from *B. aerogenes* by its inability to ferment glycerol.

The characterization of *B. communior* as salicin negative is probably untenable. The term *B. coli-communior* was first employed by Durham to describe members of the colon group which fermented sucrose and which were motile. Later Ford recognized it as a species *B. communior.*

Of 77 motile sucrose fermenting bacilli of the coli section, 56 (73%) were found by the writer to be salicin fermenters. It is felt therefore that *Bact. communior* should not be described as a salicin non-fermenter.

Where the principle of correlation has been employed the best correlated character has apparently been picked out by inspection of the data. Inspection is a tedious and difficult procedure, entirely inapplicable where the number of characters considered is large, and it does not permit of a concise statement of the degree of correlation which exists between differ-

ent reactions. The author feels that considerable information in an abstract, concise, and workable form may be obtained from a study of the coefficients of correlation.

The Coefficient of Correlation. Where we are concerned merely with the presence or absence of characters the coefficient of correlation between any two characters may easily be determined. Suppose that it is desired to know if the characters X and Y are correlated and that a study of a number of organisms showed that 'a' cultures are positive for both X and Y; 'b' organisms positive for X but negative for Y; 'c' cultures are negative for X and positive for Y; and 'd' strains are negative for both X and Y. The distribution of the organisms is first tabulated thus:

X \ Y	+	−
+	a	b
−	c	d

The degree of association, or the coefficient of correlation, is then expressed, according to Yule, by the formula

$$\frac{ad-bc}{ad+bc} \qquad (1)$$

If 'ad' is equal to 'bc' the coefficient becomes $\frac{0}{ad+bc}$ or 0; which indicates that there is no correlation whatever. If either 'b' or 'c' is zero the formula becomes $\frac{ad}{ad}=1$; indicating a perfect positive correlation. If 'a' or 'd' is zero then we have $\frac{-bc}{bc}=-1$; showing a perfect negative correlation. It should be observed that an absolute positive correlation exists in reality only if both 'b' and 'c' are zero and an absolute negative correlation when both 'a' and 'd' are zero. In order to avoid coefficients of 1 or —1 where only one group—'a', 'b', 'c', or 'd'—is zero. Yule gives the formula

$$\frac{a\,(a+b+c+d)-(a+c)\,(a+b)}{\sqrt{(a+c)\,(b+d)\,(a+b)\,(c+d)}} \qquad (2)$$

In practice, however, a few strains are almost always found in each of the four groups and Yule suggests the use of the simpler formula (1). Some caution should therefore be employed in interpreting coefficients of 1 or —1.

It was assumed that if the coefficient between two characters is numerically greater than 0.5 they may be regarded as correlated, but if less than

M.R. \ V-P.	+	−
+	7	43
−	132	2

$\frac{7\times2-43\times132}{7\times2+43\times132}=-1.00$ Negative Correl'n

Raffinose \ Sucrose	+	−
+	89	7
−	4	82

$\frac{89\times82-4\times7}{89\times82+4\times7}=+.99$ Positive Correl'n

Dulcitol \ Salicin	+	−
+	75	22
−	39	46

$\frac{75\times46-22\times39}{75\times46+22\times39}=+.61$ Partial Correl'n

Dulcitol \ Indol	+	−
+	87	10
−	76	9

$\frac{87\times9-76\times10}{87\times9+76\times10}=+.02$ No Correlation

0.3 there is probably no association. A few examples of correlation coefficients actually obtained in the course of this study are given to illustrate the method of calculation.

The principle of correlation should not be applied indiscriminately to collections of data for systematic purposes. Certain characters and properties have been universally accepted as reliable and appropriate for bacterial differentiation; thus, staining reactions such as the Gram and acid fast stains, spore formation, and aerobiosis and anaerobiosis, hardly need to be bolstered up by correlation with other characters to justify their taxonomic value. On the other hand the significance of such characters as motility, indol production, and fermentation of certain substances, is still debatable.

Motility is regarded by many as a highly variable property. Perhaps it is in reality a reliable morphological difference. Certainly if it could be shown that this character goes hand in hand with several others, more reliance and attention should and would be given to motility. The same is true of the indol test. In dealing with gas formation from carbohydrates, alcohols, or polysaccharids, the question naturally arises as to which substance should be given preference for subdivision, or whether all are to be considered of equal taxonomic value. The lack of a criterion for determining the most significant fermentable substances has led to considerable confusion. It has already been pointed out how subdivision on every character studied results in an infinite number of varieties. Where we are dealing with a number of characters each of which is assumed to be of equal taxonomic significance, it would certainly be desirable and advantageous to subdivide on that character which gives the greatest amount of information as to the manner in which the resulting subgroups react with respect to other characters. It is under such circumstances that the principle of correlation of characters may be legitimately, conveniently, and advantageously employed.

The Method of Selecting the Best Correlating Character. The following example will illustrate the method of selecting the best correlated character for the purpose of subdivision of a group of organisms. Let us take for instance a group of 89 strains of the coli section, which were found to be non-fermenters of sucrose, and which it is required to further divide on one of the following characters:—motility, indol production, dulcitol, glycerol, or salicin fermentation. Tabulation is first made, as indicated below, so as to show the relation of each character to every other character and also to facilitate the calculation of correlation coefficient which are then determined for each pair of characters and recorded as indicated in Table XVIII.

For subdivision that character is selected which gives the highest coefficient of correlation with the greatest number of other characters. Thus, in the group under consideration, motility is not well correlated with any other character. Dulcitol and glycerol each have a high correlation coefficient with salicin but not with any other character. Salicin fermentation, on the other hand, is well correlated with three characters—glycerol,

TABLE XVIII. (a) SHOWING CORRELATION OF CHARACTERS AMONG 89 SUCROSE NEGATIVE STRAINS OF THE COLI SECTION.

		Motility		Indol		Dulcitol		Glycerol		Salicin	
		+	−	+	−	+	−	+	−	+	−
Motility	+	53		45	8	22	31	43	10	33	20
	−		36	34	2	11	25	26	10	18	18
Indol	+	45	34	79		29	50	61	18	51	28
	−	8	2		10	4	6	8	2		10
Dulcitol	+	22	11	29	4	33		27	6	28	5
	−	31	25	50	6		56	42	14	23	33
Glycerol	+	43	26	61	8	27	42	69		48	21
	−	10	10	18	2	6	14		20	3	17
Salicin	+	33	18	51		28	23	48	3	51	
	−	20	18	28	10	5	33	21	17		38

TABLE XVIII. (b) COEFFICIENTS OF CORRELATION FOR EACH PAIR OF CHARACTERS IN TABLE XVIII. (a).

	Motility	Indol	Dulcitol	Glycerol	Salicin
Motility		−.50	+.22	+.25	+.25
Indol	−.50		−.07	−.08	+1.00
Dulcitol	+.22	-+.07		+.20	+.78
Glycerol	+.25	−.08	+.20		+.86
Salicin	+.25	+1.00	+.78	+.86	

dulcitol, and indol, showing cœfficients of +0.86, +0.78, and +1.0 respectively. Subdivision is therefore made upon salicin.

For each of the resulting subgroups new correlation tables are made and further subdivision again carried out on the best correlated character. A point is very quickly reached where further subdivision, upon correlated characters, is no longer feasible. These groups are regarded as species and to each was assigned, as far as possible, the name of the MacConkey variety which it most resembled.

Levine's Classification. The following classification is suggested by the author, based upon a study of 333 strains obtained from soil, sewage, and the feces of man, horse, sheep, pig, and cow.

The characters employed are the methyl-red and Voges-Proskauer reactions, indol production, motility, gelatin liquefaction and gas formation from sucrose, raffinose, dulcitol, glycerol, salicin, dextrin, inulin and corn

starch. Other fermentable substances—lactose, maltose, galactose and mannitol—were also observed but as these substances were all attacked with gas formation they need not be considered.

As was discussed in detail in Chapter II, the investigations of Harden, Smith, Rogers and others on carbohydrate and nitrogen metabolism have demonstrated adequately and conclusively that the colon group includes two distinct subgroups, the methyl-red positive-Voges Proskauer negative-uric acid negative group designated herein as the coli section, and methyl red negative Voges Proskauer positive-uric acid positive group or aerogenes section. These two main sections are recognized and each is subdivided into species on the basis of correlated characters as described above.

KEY TO THE MORE IMPORTANT SPECIES OF THE COLON GROUP.

The colon group includes all non sporing Gram negative short rods, fermenting glucose and lactose with acid and gas production and which grow aerobically.

I. Not producing acetyl methyl carbinol (Voges Proskauer reaction negative); acid to methyl red, and carbon dioxide and hydrogen produced in approximately equal volumes from glucose. Cannot utilize uric acid as a source of nitrogen.

COLI SECTION

- A. Sucrose not attacked.
  - 1. Salicin fermented with acid and gas. — 1. *Bact. coli*
  - 2. Salicin not attacked. — 2. *Bact. acidi-lactici*
- B. Sucrose fermented with acid and gas.
  - 1. Motile. — 3. *Bact. communior*
  - 2. Non-motile.
    - a. Salicin fermented with acid and gas. — 4. *Bact. neapolitanum*
    - b. Salicin not fermented. — 5. *Bact. coscoroba*

II. Producing acetyl methyl carbinol (Voges Proskauer reaction positive); alkaline to methyl red, and forms two or more times as much carbon dioxide as hydrogen from glucose. Capable of utilizing uric acid as a source of nitrogen.

AEROGENES SECTION

- A. Glycerol and starch fermented with acid and gas formation; non motile, gelatin not liquefied. — 6. *Bact. aerogenes*
- B. Glycerol and starch not fermented; motile, gelatin liquefied. — 7. *Bact. cloacae*

In the 1917 and 1920 Standard Methods of Water Analysis the coli section is not differentiated into species. The aerogenes section is subdivided on the fermentation of adonitol into a fermenting variety supposedly of fecal origin and a non-fermenting variety which is regarded as of non-fecal origin. The value of adonitol for this distinction has not as yet been adequately investigated and is not generally accepted.

IV. THE DETECTION OF THE COLON GROUP IN WATER.

Various methods have been suggested for the isolation of members of the colon group. They are all based on the principle that all members of this group of organisms are capable of decomposing lactose with acid and gas production. Media are employed which contain lactose and some indicator to show whether fermentation has taken place.

Isolation may be accomplished (1) directly by plating on solid differential media or (2) indirectly on solid differential media, after preliminary enrichment in some fluid medium.

The mediums most commonlly employed for direct isolation are:

Litmus lactose agar.
Phenolated litmus lactose agar.
Endo agar.
Conradi Dragalski agar.
MacConkey agar.

Litmus lactose agar consists of nutriment agar, neutral to phenolphthalein, which contains sufficient litmus or azolitmin to give a distinct blue color. Fermentation of lactose with the production of acid is indicated by the formation of red colonies.

The technique for direct isolation is merely to place definite quantities of the test sample (1.0 c. c., 0.1 c. c., 0.01 c. c., etc) into petri dishes to which are added about 10 c. c. of litmus lactose agar. (In some laboratories the litmus is added to the petri dish and then lactose agar poured in.) The plates are incubated at 37 degrees to 40 degrees C. for 24 hours after which members of the colon group will appear as red colonies on a blue background. As there are other organisms, particularly the streptococci, which are capable of fermenting lactose with acid production, the mere appearance of acid colonies is not absolute proof of the presence of the colon group. In the process of sterilization, lactose is sometimes broken down so that organisms other than the colon group may then produce slightly acid reactions. They must therefore be examined further. Suspicious colonies are fished to agar and a Gram stain is made to insure that the organism is a Gram negative non-spore-forming rod. From the agar slant various other tests (fermentation of carbohydrates, gelatin liquefaction, milk coagulation, the V. P., methyl red, etc.) may be carried out.

Acid produced by the growth of fermenting forms diffuses through the medium, often coloring considerable portions of the plate, making it extremely difficult to detect the acid producing colony. The medium exerts very little inhibitory action and overgrowths of non-colon forms are not infrequent. These disadvantages may be overcome to some extent by (1) the use of porous tops to prevent spreaders, (2) by increasing the concentration of agar to 3 percent thereby diminishing considerably the diffusion of acid, and (3) by the addition of selective antiseptics to inhibit the growth of forms other than the colon group. The use of the higher temperature (37 degrees C.) and preferably 40 degrees C., exerts

some inhibitory action on the ordinary water forms and many soil forms. This may be further increased by the addition of small quantities of phenol to the agar medium. Chick found that 0.1 percent (1 part per thousand) gave very excellent results. In using phenolated media, it must always be borne in mind that there is danger of inhibiting some members of the colon group as well.

Endo agar consists of nutrient lactose agar containing 3 percent agar with basic fuchsin decolorized by sodium sulphite as an indicator. The lactose fermenting organisms produce red colonies often with a distinct metallic luster, the medium itself being colorless or slightly pink. The reaction is presumably due to the production of acid and aldehyde by the lactose fermenting organisms. These products react with the fuchsin sulphite combination liberating the fuchsin. The high concentration of agar serves to check diffusion of acid and also to eliminate many of the water forms. It was thought at one time that the indicator itself exerted inhibitory action but this does not seem to have been adequately proven.

Conradi-Drigalski agar consists of litmus lactose agar to which has been added some nutrose and crystal violet. The crystal violet checks the growth of many forms particularly the streptococci but is supposedly non-inhibitory for the colon group in the concentration employed. Both the Endo and the Conradi Drigalski have been found very valuable for the isolation of the para typhoid and typhoid bacilli from stools but they have never found favor in the United States although they are the media of preference in Germany for direct isolation of the colon group from water.

MacConkey agar (also known under the name Rebipelagar) consists of peptone lactose agar with 0.5 percent bile salts (sodium glycocholate and taurocholate), the function of the latter being to check saprophytic forms. The inhibitory action of bile is well illustrated in Table XX.

TABLE XX. SELECTIVE ACTION OF BILE SALTS.
(After Jackson 1906)

	Bacteria per. c. c.			
	Uncontaminated well	Contaminated pond	Suspension of feces	Suspension of feces
Gelatin 20°	920	2700	350.000	900.000
Agar 37°	25	170	450.000	900,000
Bile Agar *37°	14	43	300,000	900.000
Bile Agar 37°	0	16	60.000	900.000
Lactose Bile Agar *37°	0	25	250,000	675,000
Lactose Bile Agar *37°	0	17	250,000	600,000

*Bile diluted, 1:1.

where it will be noticed that the bile salts exerted a much greater restraining action on forms generally found in water than on the organisms (almost exclusively *Bact. coli*) present in a suspension of feces. The

indicator in the Rebipelagar is neutral red. Acid producing colonies are distinctly red. This medium is very extensively used in England but has not found favor in the United States for, as will be shown later in the discussion of the presumptive test for *Bact. coli*, there is considerable evidence that the bile salts may inhibit some strains of *Bact. coli.* Reference to the foregoing table shows that in one of the fecal suspensions a count of 450,000 on agar was reduced to 60,000 by bile.

The isolation of the colon group by direct plating is recommended for highly polluted waters and sewage where the number of coli strains are quite large, being present in one c. c. or less. If, however, it is necessary to employ large samples (5 c. c. or 10 c. c.) as is essential in routine water work, it becomes extremely inconvenient to plate out. Furthermore, if the incidence of the colon group is small compared to other forms, they may be missed. Although theoretically the colon colonies are clearly differentiated from others, it is the practical experience of all investigators that when growing on a plate with a large number of alkali formers, the acid of *Bact. coli* and related forms may be neutralized, and the characteristic reaction thereby masked, so that a colon colony may thus escape detection. On the other hand, in the process of sterilization of medium the lactose may be decomposed in part and it will then be found that some soil forms will produce an acid reaction on solid media. Acid production on any of the solid media described above can therefore not be relied upon to always indicate the colon group, and so further tests must be resorted to as has been mentioned in discussing litmus lactose agar.

For the isolation from relatively large quantities of water, or for an admixture with numerous other forms, the best results are obtained by the indirect or so-called preliminary enrichment method. The procedure consists of three steps:

1. Growth in a favorable liquid medium containing some constituent which will indicate the probable presence of the colon group. This is spoken of as a preliminary enrichment tube and is often employed as a presumptive test.

2. Isolation from this preliminary enrichment tube on some one of the solid media described above. If typical colonies are formed the presence of members of the colon group is considered partially confirmed.

3. Suspicious colonies on (2) are examined, by fishing to agar and then testing for coagulation of litmus milk, gas in lactose broth, V. P., etc. This would constitute a completely confirmed test.

The media most commonly employed for preliminary enrichment are:

Glucose broth
Phenol broth
Liver broth
Lactose broth
Lactose bile salts broth
Eijkman test (glucose peptone water at 46° C.)
Lactose peptone bile

Glucose broth consists of ordinary broth, neutral to phenolphthalein, with 1 percent glucose. This medium was the one most commonly employed in the United States prior to 1906. The probable presence of the colon group is indicated by the production of gas after 24 to 48 hours incubation at body temperature.

Phenol broth consists of ordinary broth containing 0.1 percent carbolic acid which exerts a selective antiseptic action on the normal water bacteria thereby supposedly favoring the growth of the colon types. This medium is employed by the French, but it has been observed that the phenol exerts an antiseptic action on some members of the colon group and that its employment is particularly objectionable with water of fairly good quality.

Liver broth has been found particularly efficacious for isolation of colon types and in 1912 the Standard Methods for Water Analysis of the A. P. H. A. recommended that, "If a further study of all gas forming bacteria, including attentuated forms, is desirable, then liver broth should be employed in preference to the usual dextrose broth, as it gives a larger number of attentuated forms, has better rejuvenating powers, and gives fewer anomalies, and greater and more rapid gas production." For routine water work, however, liver broth has not found favor.

Since 1906, there has been a tendency to substitute lactose for dextrose in the preliminary enrichment mediums. It is quite surprising that this was not done sooner considering that the essential characteristic of the colon group is lactose fermentation.

Lactose broth, which is nutrient broth containing 0.5 to 1.0 percent lactose, is most commonly employed in the United States at the present time and is the medium recommended in the Standard Methods of Water Analysis of the A. P. H. A. of 1917 and 1920. It is supplanting the lactose peptone bile medium of Jackson, which was extensively used between 1912 and 1917. In both of these mediums the probable presence of the colon group is indicated by gas production from the lactose.

Lactose bile salt broth of MacConkey consists of 0.5 percent lactose, 2% peptone, 0.5 percent sodium taurocholate with neutral red as an indicator. The colon group will produce gas and acid indicated by red coloration of the liquid after 24 to 48 hours at 37° C.

Technique for Isolation. The steps in the isolation of the colon group from water may be summarized as follows:

(1) Place various quantities of the water under examination (10 c. c., 1 c. c., 0.1 c. c., etc.) into Smith or Durham fermentation tubes containing the preliminary enrichment medium.

(2) Incubate at the body temperature (37° C.) for 24 hours. If gas is formed, it is taken as presumptive evidence that *Bact. coli* or its close allies are present.

(3) The next step is then to isolate these organisms on some one of the solid media enumerated above. This may be done in one of the following ways:

Method 1. Barely touch the liquid in the preliminary enrichment tube, showing gas, with the point of a platinum needle, wash off this needle in a tube of melted litmus lactose or Endo agar and pour into a petri dish. Incubate at the body temperature for 24 hours.

Method 2. Pour L. L. A., Endo or other differential agar into petri dishes and allow them to solidify and dry in the incubator. Dip a small loop into a positive preliminary enrichment tube and wash it off in a tube of sterile salt solution. Place a loop of this wash water in the center of a plate containing the differential medium and spread it over the surface with the aid of a sterile glass rod bent at right angles. Incubate 20 to 24 hours at body temperature.

Method 3. Dip a platinum needle, whose end is bent at an angle of about 120°, into the positive preliminary enrichment tube, stab into a plate containing the differential medium (to remove the excess organisms) and then make a series of streaks on this plate about a quarter of an inch apart, taking care to always streak in the same direction and to lift the needle at the end of each stroke. With a little practice, very excellent isolations may be obtained by this simple method. This was first demonstrated to the author by Mr. Greenfield of the Illinois Water Survey and it has proved very satisfactory. Incubation is, as before, at body temperature for 24 hours.

TABLE XXI. RELATIVE GROWTH OF *BACT. COLI* AND SEWAGE STREPTOCOCCI FROM POLLUTED WATER IN GLUCOSE BROTH.
(After Prescott and Baker 1904)

Sample No.	Initial number of red colonies per c. c. on L. L. A.	Type	Number found in million per c. c. after various periods of growth					First gas noticed after
			11 hrs.	16 hrs.	23 hrs.	39 hrs.	63 hrs.	
2	10	*Bact. coli*	20	76	150	0	0	11 hrs.
		Strep.	0	25	85	420	0	
7	35	*Bact. coli*	130	140	200	20	8	10 hrs.
		Strep.	0	20	110	400	45	
8	460	*Bact. coli*	332	420	405	24	0	6 hrs.
		Strep.	0	210	350	105	150	

If characteristic colonies are present on these agar plates the test is regarded as partially confirmed for the colon group. For further confirmation, it would be necessary merely to fish a well isolated colony and

to determine its Gram characteristics and other reactions, particularly fermentation of lactose broth, as previously explained.

The objections to the preliminary enrichment method are:

(1) It is difficult to estimate the incidence of the colon forms for by employing 1c. c., 0.1 c. c., etc., there is considerable error introduced in calculating the number of organisms present in a unit volume of water.

(2) There is a danger of loss of the colon forms which may be killed off before isolation is attempted either by (a) products of their own metabolism or (b) by over-growths of other organisms.

In glucose broth inoculated with a mixture of *Bact. coli* and *Streptococci,* the former usually predominated for the first 24 hours, but thereafter the number of *Streptococci* was considerably greater, in some instances having completely choked out or killed the colon types in 39 hours.

The danger of losing colon strains due to overgrowth and death may be minimized considerably, if not completely eliminated, by (1) reducing the concentration of fermentable substances to 0.5 percent (as recommended by Burling and Levine) and (2) attempting isolation at the earliest possible time (after 12 to 24 hours or at the first appearance of gas). It will be noticed from Table XXI. that at the first appearance of gas the colon forms were far in excess of the *Streptococci,* in fact practically in pure culture. With these precautions the preliminary enrichment method will be found very reliable and convenient.

THE PRESUMPTIVE TESTS

The method for isolation of members of the colon group described above, comprising as it does the reaction of the isolated organism in milk, production of indol, reduction of nitrates, gelatin liquefaction, and motility was in general use between 1905 and 1910. The method, however, was soon found to be tedious and inconvenient for routine work. The time required for such a complete analysis was at least nine days, whereas for practical purposes it is desirable to have a report available within 24 to 48 hours. Furthermore, there is no evidence that the determination of the characters enumerated shed any light upon the sanitary significance of the bacilli isolated. For routine water work, it is essential to have some test which is simple and rapid and which can be relied upon as a reasonable index of the probable presence of the colon group in a large proportion of instances. Such a test is known as a **presumptive test** for the colon group. From his wide experience with the glucose broth preliminary enrichment method, Whipple in 1903 observed that a considerable portion of tubes showing characteristic fermentation were found, on further examination, to contain colon-like organisms. He therefore suggested that dextrose broth alone might be employed as a rapid presumptive test of the probable presence of *Bact. coli.* The media which have been and are most commonly employed for the presumptive test are (1) glucose broth, (2) lactose peptone bile, and (3) lactose broth. Other media have also been suggested, as for example the Eijkman test (glucose peptone

water at 46° C.) and neutral red broth, but these are not employed to any extent in the United States and will not be considered further.

The Glucose Broth Presumptive Test. The test consists of the determination of the amount of gas and the ratio of hydrogen to carbon dioxide in glucose broth after 24 hours incubation at the body temperature.

Production of 25 to 70 percent gas, one-third of which is carbon dioxide and the remainder hydrogen (that is $H/CO_2=2$ to 1) is considered a "typical" test and is regarded as indicating the presence of *Bact. coli* or closely related bacilli. Thus Irons stated that when the proportion of carbon dioxide is approximately 33 percent *Bact. coli commune* is almost invariably present.

If the amount of gas is (a) between 10 and 25 percent, (b) more than 70 per cent or (c) if the proportion of carbon dioxide is more than 40 percent of the total volume of gas formed, the reaction is considered "atypical" and the probable presence of the colon group is doubtful.

If there is less than 10 percent gas, or if no gas is formed at all, the probability of isolation of the colon group is very slight and the test is considered negative.

The early studies indicated that the colon group was successfully isolated from about 70 percent of positive presumptive tests. More recent and detailed studies have shown, however, that the proportion of presumptive tests confirmed by further observation is not constant but is effected by the character, treatment, etc. of the water under examination and the season of the year.

Objections to the Glucose Broth Presumptive Test. The objections to the glucose broth presumptive test may be summarized as follows:

1. The volume of gas produced is not a significant criterion of the probable presence of the *Bact. coli* or its close allies.
2. The gas ratio, as determined in the Smith tube, is unreliable.
3. There are many glucose fermenting species which are incapable of attacking lactose.
4. The reliability of the glucose presumptive test varies with (a) the season of the year, and (b) differs for raw and treated waters.
5. The glucose broth medium is particularly subject to overgrowths.

That 25 to 75 per cent gas is not necessarily characteristic of the colon group and that even pure cultures produce as little as 10 percent gas has been demonstrated by Fuller and Ferguson, Longley and Batton, Hale and Melia, and others. They also have shown that the so-called 'typical' gas ratio is not at all characteristic of the colon group. Thus of 818 tubes of sterilized water, inoculated with *Bact. coli* but containing no other gas former, only 474 or 58 percent gave the typical gas volume and gas ratio. The errors inherent in the gas ratio as determined in the Smith tube have already been discussed in detail in Chapter II.

Fermentation of glucose with gas production is not a very reliable indication that the organism will also ferment lactose. There are many species of bacteria capable of attacking the monosaccharids, but which

are inert as respects the more complex disaccarid lactose. Thus Clark and Gage in 1903 pointed out that there were 58 well described species which gave the presumptive test in dextrose broth of which 23 are widely separated from the colon group and since then many other glucose-positive-lactose-negative forms have been described.

Prescott and Winslow call attention to the seasonal variation in the reliability of this presumptive test noting that Fromme (1910) isolated colon bacilli from 87 percent of positive glucose broth tubes in the fall and winter (Oct. to Mar.) but only 66 percent from similar tubes in the spring and summer (Apr. to Sept.)

In stored or filtered waters the incidence of glucose fermenters which do not attack lactose is considerably greater than in raw waters. The reports of Houston for 1909-11, which are summarized below, show that only 9.5 percent of coli-like bacteria isolated from raw river water after preliminary enrichment in glucose broth failed to ferment lactose but that with filtered and ground waters 38.3 percent of the isolated bacilli did not attack this disaccharid.

TABLE XXII. GLUCOSE AND LACTOSE FERMENTATION BY COLI-LIKE BACTERIA FROM RAW AND FILTERED WATERS.
(After Houston 1909 to 1911)

	Raw River waters	Filtered waters*
Number of Strains	7,657	10,620
Glucose+; Lactose—	9.5%	38.3%
Glucose+; Lactose+	90.5%	61.7%

*Includes ground waters.

TABLE XXIII. EFFECT OF NATURAL AND ARTIFICIAL PURIFICATION ON INCIDENCE OF GLUCOSE AND LACTOSE FERMENTING BACILLI IN PULTA WATER.
(After Clemesha 1912)

	Intake		Settled		Filtered	
	*No. of strains	% Lactose negative	*No. of strains	% Lactose negative	*No. of strains	% Lactose negative
October (Heavy rain)	40	40.0	40	50.0	40	50
November (River muddy but no rain locally)	80	31.2	80	16.2	80	48.7
December (River clearing)	60	70.0	60	85.0	60	91.6
January (River clear)	100	61.0	100	71.0	60	100.0
February (River clear)	80	93.7	80	98.7	60	98.2

*All glucose fermenters.

Clemesha (1912) has made numerous observations on the relative incidence of glucose and lactose fermenting bacilli in waters of India subjected to various periods of storage, exposure to sunlight, and filtration. In Table XXIII. taken from his book on the bacteriology of Surface Waters in the Tropics are shown the effects of treatment and the influence of self purification on the relative incidence of glucose fermenting bacilli which are not lactose fractors.

The increased incidence of glucose positive-lactose negative organisms in waters during dry weather is particularly striking. Thus during the rainy month of October 40 to 50 percent of the strains studied were of this type, the number gradually increasing as the river cleared and the forces of natural purification (sedimentation, sunlight, etc.) manifested themselves until in February, 93.7 to 98.2 percent of the glucose fermenting strains were non-lactose fractors. The glucose broth presumptive test is therefore particularly unreliable as an index of the colon group when dealing with treated and stored waters.

Clemesha attributes the marked increase of glucose +, lactose —, bacilli in India stored waters to the presence of an organism which he designates *Bacillus P.* This bacillus is widely distributed in nature, extremely resistant and capable of multiplying in water.

The important characteristics of the organism are given below:

Morphology, etc. Gram positive, motile bacillus.

Litmus Milk. Slight acidity on top (occasionally). No coagulation.

Indol. Negative.

Gelatin. Not liquefied in ten days.

Fermentation. Glucose acid and gas.
Sucrose acid and gas.
Lactose. Faint trace of acid on long cultivation in laboratory.

Dulcitol, Mannitol, Adonitol, Inulin } Neither acid nor gas

Voges Proskauer Reaction. Strongly positive.

Bacillus P. of Clemesha resembles the *B. proteus* but differs from it in being Gram +, and giving a positive Voges Proskauer reaction.

Not only is gas production in glucose broth a poor index of probable presence of *Bact. coli* but the organism may be present even in the absence of gas formation, particularly if the water has a high bacterial count or contains streptococci. It is not infrequently observed with this medium that small samples of water (1 c. c. or 0.1 c. c.) may be positive whereas larger portions (10 c. c.) may be negative. Upon considering that there are numerous organisms capable of attacking glucose with acid production, this choking off of the colon bacilli may be readily explained by the inhibiting action of the acids formed.

This lack of constant relation between the dextrose broth fermentation and the actual presence of the colon group has resulted in a gradual abandonment of the **glucose broth presumptive test** in favor of media containing lactose.

The Lactose Peptone Bile Presumptive Test. The use of bile containing lactose was first suggested in this country by Jackson in 1906. He recommended fresh ox bile containing 1 percent lactose but the inclusion of 1 percent peptone was soon found to be desirable. The medium is prepared by adding 1 percent peptone and 1 percent lactose to fresh undiluted ox gall (or a 10% solution of dried ox gall) which is placed in fermentation tubes and sterilized in the usual manner. He found, however, that a longer period of incubation than is employed in dextrose broth presumptive test is necessary and recommended 72 hours. For ordinary work a period of 48 hours is ample. In this medium, the production of 10 percent or more gas is considered a **positive presumptive test.** The proportion of positive presumptive tests found on further study to contain the colon group is very high, Stokes and Stoner reporting 95 and Cumming 87 percent. Its superiority to dextrose broth is quite marked. Thus Prescott and Winslow, from a study of 176 surface waters in eastern Massachusetts, obtained 120 positive fermentations with dextrose broth and 78 with lactose bile: but *Bact. coli* was isolated from only 70 (58%) of the dextrose as compared with 64 (82%) of the positive bile tubes. Reliance on the dextrose broth presumptive test would have introduced an error of 70 percent as compared to an error of 11 percent for the lactose bile medium.

The superiority of lactose bile, as a presumptive test, is evident. This is due partly to the selective antiseptic action of the bile salts but also to a considerable extent to the use of lactose which automatically eliminates the dextrose positive-lactose negative organisms. It should also be noted that the presence of lactose may indirectly inhibit non-lactose fermenters for by stimulating the growth of the colon group, the resultant acid production through fermentation of the disaccharid may in turn inhibit markedly the growth of water forms. In lactose media the probability of the colon group being overgrown is considerably reduced as compared with glucose media where acid may be produced by many other bacilli resulting in the retardation of the colon group itself.

Consideration of the example cited above indicates, however, that although lactose bile is superior as a presumptive test, the actual number of positive isolations with this medium (64) was about 8.5 percent less than was obtained with dextrose broth (70). Apparently therefore the inhibitory action of the bile is not restricted to water forms, but is exerted to some extent, on the colon group as well.

The Committee on Standard Methods (1912) suggested that this inhibitory action is exerted on the weaker members of the colon group, strains which have become attenuated, so to speak, and which are indicative only of remote pollution and therefore of apparently little significance. Thus they say, "In the interpretation of the sanitary quality of a water, it

is best to discount the presence of attenuated *Bact. coli* and to be sure to obtain all vigorous types. The lactose bile medium accomplishes both these objects."

This contention, that bile affects the weaker strains, must be regarded merely as an assumption, for it is not substantiated by actual observations. Jordan, in a careful study of the subject, concludes that there is no relation between attenuation and the antiseptic action of the bile. He found that freshly isolated strains were inhibited to as great or even greater degree than old strains. Cumming, in a comparison of lactose bile and lactose broth, found that with sewage preliminary enrichment in lactose bile yielded only 25 percent as many colon forms as were obtained with lactose broth. In a similar study with river water, the bile method gave 50 to 70 percent as many colon organisms as the broth. These results would indicate quite the converse of the contention of the 1912 report of the Committee on Standard Methods that "Attenuated *B. coli* does not represent recent contamination, and all *B. coli* not attenuated grow readily in lactose bile."

The 1917 and 1920 reports of the Committee on Standard Methods of Water Analysis recommend the use of lactose broth for preliminary enrichment and the presumptive test on the ground tha it gives a higher total number of positive isolations. The concensus of opinion at present seems to be in favor of the substitution of broth for bile.

Lactose Broth vs. Lactose Bile. Jordan in 1913 called attention to the greater proportion of successful isolations of *Bact. coli* with lactose broth. Of forty—5 c. c. portions of Lake Michigan water inoculated into lactose broth and lactose bile media, colon bacilli were isolated from 42 percent of the former and only 30 percent of the latter. Similarly one hundred and fifty—1 c. c. samples yielded 31 percent positive isolations with lactose broth compared to 22 percent when lactose bile was employed for preliminary enrichment.

Creel in a study of drinking waters on railroad trains reports the following frequencies of the colon group in comparative tests:

Water samples containing colon group	91
Positive from lactose bile only	18
Positive from lactose broth only	45
Positive from both media	27

The inhibitory action of the bile is very marked. About 50 percent of the colon bacilli isolation would have been lost if reliance had been placed on lactose peptone bile alone.

Similarly Dr. Cumming in the Potomac River studies calculates the number of colon bacilli to be 84 per c. c. by lactose broth enrichment followed by confirmatory tests as compared to 47 when lactose bile was employed and followed by confirmatory tests, again indicating that about half of the colon bacilli were lost when preliminary enrichment was carried out in the bile medium.

Obst comes to the same conclusion. Houser of the Cincinnati Water Supply prefers the broth medium. Ritter in a detailed comparison of

both media concludes that if both lactose broth and lactose bile are positive in 24 hours, the probability of the presence of the colon group is very great (about 98% confirmed by subsequent tests) and that, in general, if there is fermentation in both bile and broth media the presumptive test is reliable in about 75 percent, but if either medium is negative, then the proportion of positively confirmed tubes is very much less, even as low as 25 to 45%. This high proportion of positively confirmed presumptive tests, when both broth and bile are positive, may find a ready explanation in the observations of Creel. Most of the spurious presumptive tests are due to the presence of spore forming anaerobic lactose fermenters. Creel found that some of these anaerobes are capable of growing in broth but not in bile. He calls this "Group A" whereas another "Group B" will grow readily in bile but not in broth. Thus a positive test in both lactose broth and lactose bile automatically eliminates both of these anaerobic spore formers and thereby accounts for the high proportion of positively confirmed tests.

Opposed to the views expressed above is that of Hale who recently (1917) stated that from a careful detailed, and extensive comparative study on the two media at the Mount Prospect Laboratory, he considers the results were all in favor of bile, that gas formation was more rapid, produced in larger amounts, and that the *B. clostridium welchii* types (an aerobic spore formers) were less frequent. Probably his experiences may be explained by the fact that, in these experiments, he employed a 5 percent dried bile, whereas the observations of other investigators were concerned with the original medium, containing either undiluted ox gall or 10 percent of the dried bile.

The work of Salter and the author in the Laboratory of the Iowa Engineering Experiment Station indicated quite clearly that bile may inhibit or stimulate multiplication of *Bact. coli*, depending upon the concentration of bile salts. With less than 0.5 percent bile salts a stimulating action on the rate of multiplication was observed, whereas higher concentrations were markedly inhibitory. The effect was studied by observing changes in the generation time (See Table XXIV.).

Since bacteria divide by simple fission, the number of organisms present at any time in an actively growing culture may be expressed thus: $b = B \times 2^n$

Where "B" is the initial number of bacteria
"n" the number of generations
"b" the number of bacteria after "n" generations

If the time elapsed is "t" then "g" the generation time—equals $\frac{t}{n}$ or "n" equals $\frac{t}{g}$.

$$\text{Thus } b = B \times 2^n = B \times 2^{\frac{t}{g}}$$

$$g = \frac{t \log 2}{\log b - \log B}$$

TABLE XXIV. EFFECT OF CONCENTRATION OF COMMERCIAL BILE SALTS ON GROWTH OF *BACT. COLI* IN 0.5 PERCENT PEPTONE WATER.
(Temp. of incubation 37° C.)

Concentration of bile salts	Bacteria per c. c. After				Average generation time in minutes
	0 hrs.	2 hrs.	4 hrs.	8 hrs.	
Control 0.0%	124	410	12,800	14.600.000	28.7
0.1%	124	595	17,900	23.700.000	27.5
0.2%	124	515	17.900	36,000,000	26.6
0.3%	124	450	17,000	24,700,000	27.7
0.5%	124	520	20,200	21,500,000	27.8
0.7%	124	425	13,400	10.800,000	29.5
1.0%	124	435	7,150	1,650,000	35.4

The experience of Hale, that Cl. Welchii forms were less frequently encountered in bile than in broth, may merely mean that in the waters he worked with Creel's anaerobe of "Group A" were more frequent than "Group B". In other laboratories, the reverse might be true.

The relative merits of lactose broth and lactose bile may be summed up as follows:

1. Lactose bile is a more reliable presumptive test but a greater proportion of the colon group may be detected by preliminary enrichment in lactose broth.

2. If the proper concentration of bile salts could be determined the bile medium would probably be preferable. For the present, considering the difficulty of obtaining bile of constant composition or the chemically pure salts, and in view of our insufficient knowledge as to the optimum concentration of bile salts, it seems best to employ lactose broth as a more uniform medium may thus be obtaincd in different laboratories. It is very probable that if a standardized evaporated bile were available a con-concentration of 1 to 2 percent in lactose peptone water would be superior to lactose broth.

THE LACTOSE BROTH PRESUMPTIVE TEST.

The use of lactose broth for preliminary enrichment and as a presumptive test has received considerable impetus through the investigations of the Public Health Service and has been accepted by the Committee on Standard Methods of Water Analysis of the A. P. H. A. (1917).

Factors Affecting the Preparation of Lactose Broth. In 1917, the Standard lactose broth consisted of 0.3 percent beef extract and 0.5 percent peptone with 1.0 percent lactose, the reaction being neutral to phenolphthalein. The medium was tubed and sterilized in the autoclave at 15 pounds (120° C.) for 15 minutes.

Hasseltine pointed out that sterilization in the autoclave was objectionable as it brought about a breaking down of the lactose resulting in a considerable increase in the number of unconfirmable presumptive tests as com-

pared with the lactose broth medium employed in the Public Health Service.

The Public Health Service lactose broth is made as follows: Nutrient broth neutral to phenophthalein is prepared in bulk and sterilized in the autoclave. A sufficient quantity of a 20 percent solution of lactose in distilled water, previously sterilized in the Arnold for an hour and a half, is then added to make a concentration of 1 percent. The medium is then distributed aseptically into sterile Smith fermentation tubes, which are then heated in the Arnold for 30 minutes.

Hasseltine found that the "Standard" lactose broth gave 73 percent more positive fermentations and yielded 10 percent fewer confirmations than the Public Health Service medium. With reference to these observations, it should be pointed out, however, that in the preparation of the Standard broth, the period of sterilization was quite prolonged. He states that the broth was in the autoclave for about an hour (25 minutes to raise the pressure to 15 pounds, 15 minutes at that pressure, and about 20 minutes to allow the pressure to fall sufficiently to permit opening the autoclave without blowing out or wetting the stoppers). In the autoclave in which steam must be generated this prolonged period is of course probably necessary but in autoclaves provided with pressure steam, the entire period of sterilization may be reduced to 25 or at most 30 minutes; with such an instrument, the very significant objections raised by Hasseltine do not apply. In fact, Mudge, in a careful study of the relative effects of temperature and pressure as compared with time of exposure, concludes that the time factor is the more vital one in the decomposition of lactose. He maintains that heating in streaming steam for three successive days, as is ordinarily done in the Arnold, will cause a greater hydrolysis of lactose and maltose than sterilization in the autoclave at 15 pounds for 15 minutes. These conclusions are based upon the relative increase in monosaccharids as indicated in Table XXV. By use of bacterial cultures confirmatory results were obtained.

TABLE XXV. EFFECT OF METHOD OF STERILIZATION ON DECOMPOSITION OF CARBOHYDRATES.

(After Mudge 1917)

Method of sterilization	Lactose	Maltose	Sucrose	Raffinose
Autoclave 15 minutes	Trace*	1	0	0
Autoclave 30 minutes	1.5*	3	0	0
Autoclave 60 minutes	2.0	Very great	Slight	0
Arnold 1st day	0	4	0	0
Arnold 2nd day	1	Very great	0	0
Arnold 3rd day	2	Very great	0	0

*Figures indicate relative degree of reduction as determined by Barfoed's method for monosaccharids.

Mudge also makes an interesting observation as to the cause of increased acidity in sterilized culture media. As is well known, a neutral

solution of amino acids become acid on the addition of neutral formaldehyde. Sugars may be considered as polymers of formaldehyde, and, as some sugars have aldehyde groups capable of reacting, it is suggested that the increased acidity in sterilized sugar media may be due to the interaction of the amino acids in the medium with the aldehyde group of the sugar. This reaction takes place very slowly and does not become evident before 30 minutes autoclaving. In Table XXVI. this contention is very clearly demonstrated. Neither asparagine, maltose, lactose, nor rafinose become acid on autoclaving, even for an hour, but if maltose or lactose are heated in the presence of asparagine a distinct acidity develops in 30 minutes, and a much more marked acidity after one hour. With raffinose, on the other hand, there is no increase in acidity. The disaccharids (maltose and lactose) contain reactable aldehyde groups whereas the trisaccrid, raffinose, does not. The contention of Mudge that it is the presence of an unstable sugar molecule together with an amino acid which gives rise to the acidity on sterilization appears very plausible.

TABLE XXVI. EFFECT OF AN AMINO ACID (ASPARAGINE) ON PRODUCTION OF ACID FROM SUGARS, BY AUTOCLAVING.

(After Mudge 1917)

Medium	Acidity (% normal) after autoclaving for		
	15 min.	30 min.	60 min.
Asparagine	0.0	0.0	0.0
Maltose	0.0	0.0	0.0
Lactose	0.0	0.0	0.0
Raffinose	0.0	0.0	0.0
Asparagine + Maltose	0.0	0.44	0.58
Asparagine + Lactose	0.0	0.34	0.64
Asparagine + Raffinose	0.0	0.0	0.0

In the revised Standard Methods for 1920, two important modifications in the preparation of lactose broth were recognized:

(1) The method of sterilization recommended for autoclaving is 15 pounds for 15 minutes provided the total time of exposure to heat is not more than a half-hour. "Otherwise a 10 percent solution of the required carbohydrate shall be made in distilled water and sterilized at 100° (for one and a half hours), and this solution shall be added to sterile nutriment broth in amounts sufficient to make a 0.5 percent solution of the carbohydrates and the mixture shall then be tubed and sterilized at 100° C. for 30 minutes, or it is permissable to add by means of a sterile pipette directly to a tube of sterile neutral broth enough of the carbohydrates to make the required 0.5 percent. The tubes so made shall be incubated at 37° C. for 24 hours, as a test for sterility."

(2) The concentration of lactose is reduced to 0.5 percent. This seems to be desirable as it tends to eliminate the danger of loss of the colon group through destruction by acid formed in fermentation.

Clemesha, in 1912, found that in peptone bile salt, neutral red broth containing 1 percent lactose, pure cultures of *Bact. coli* and *Bact. aerogenes* begin to fall off in numbers after 24 hours. Burling and Levine observed that the concentration of carbohydrates influences markedly the viability of the colon group in culture media. With 0.3 percent glucose or lactose, the number of viable organisms reached a maximum of 100 to 1,000 million in about 10 hours and remained constant for about 7 days. Increasing the amount of carbohydrates to about 0.5 percent resulted in death of over 99 percent of the organisms in a week, whereas with a concentration of 1 percent of the carbohydrates a count of 100 million, after 24 hours, was reduced to less than 10,000 (a reduction of 99.9 percent in 72 hours.)

In preparing carbohydrate media the author has used the following method with excellent results:

The broth containing 0.5 percent of the carbohydrate is distributed in sterile Durham fermentation tubes, using ordinary precautions but not necessarily strict asepsis, and autoclaved at 10 pounds for 10 to 15 minutes in a pressure steam sterilizer. Immediately after removing from the autoclave, the tubes are cooled by placing in running tap water after which they are incubated at 37° C. for 24 to 48 hours to eliminate unsterile tubes. Several tubes are inoculated with *Bact. enteritidis* or *Bact. paratyphosum* and incubated for 24 hours to detect hydrolysis as shown by gas formation.

Wagner and Monfort have recently reported that if gentian violet (1-20,000 to 1-100,000) be added to lactose broth, the medium may be prepared by a single heating in the Arnold or simply pasteurization. If further experience proves gentian violet non-inhibitory to the growth of *Bact. coli*, such a medium would be practically ideal, for not only is the danger of inversion of the lactose eliminated, but growth of the spore forming lactose fermenters is also reported to be checked.

In recording the presumptive test with lactose broth (this applies to lactose bile also), the production of 10 percent or more gas is regarded as a **positive presumptive test.** If the amount of gas is less than 10 percent the test is regarded as questionable. If there is no gas it is of course negative. Experience indicates that where the quantity of gas is more than 70 percent the fermentation is very likely due to anaerobic spore formers rather than to the colon group.

Reliability and Value of the Lactose Broth and Lactose Bile Presumptive Tests. In studies on the Ohio and Mississippi Rivers, Fuller noticed that the results with these presumptive tests were far beyond what could be explained by ordinary pollution and much higher than results obtained by a complete isolation test. Similarly in studies on the Potomac River, Cummings found these tests were reliable when pollution was excessive, but as purification progresses, the correlation between the presumptive and confirmatory tests become less marked. Using Endo agar for confirmation 92 percent of positive presumptive tests were confirmed in regions close to the source of pollution but in the vicinity of oyster beds, which were further removed from pollution, only 47.5 percent of the positive presumptive tests were found to contain members of

the colon group. The reliability of these presumptive tests therefore varies directly with the degree of contamination or inversely with the remoteness in time and distance from the source of pollution. The non-confirmed presumptive tests are due, for the most part, to lactose fermenting spore producing anaerobes which persist for a long time in water and which are probably of very little sanitary significance.

Hall and Ellefson have suggested the addition of gentian violet to lactose broth to restrict the growth of these anaerobes, and recently Meur and Harris indicate that brilliant green may be employed in lactose bile to eliminate these spurious presumptive tests. This will be considered more in detail in a subsequent chapter.

The reliability of the presumptive test also varies with the treatment which the water has received. Hauser, using lactose broth, obtained confirmations in 97.4 percent of raw water samples as compared with 86.1 percent of samples taken at the outlet from the clear water reservoir.

The effect of chlorination on the reliability of the presumptive test is particularly marked and is shown in the following able:

TABLE XXVI. SHOWING CORRELATION OF RATE OF GAS PRODUCTION WITH CONFIRMATION OF THE PRESUMPTIVE TEST FOR COLON GROUP IN LACTOSE BROTH.

Rate of Gas Production	Untreated supplies		Chlorinated supplies	
	Number of tubes showing gas	Percentage of gas tubes confirmed	Number of tubes showing gas	Percentage of gas tubes confirmed
Rapid (10 percent or more in twenty-four hours)	684	97.7	57	44.0
Moderate (less than 10 percent in twenty-four hours)	193	91.2	15	20.0
Slow (no gas in 24 hours; 10 percent or more in forty-eight hours)	276	73.2	156	6.4
Very slow (no gas in twenty-four hours; less than 10 percent in forty-eight hours)	33	45.5	26	0.0

The results indicated are based on a study of 1559 water analyses in the Advanced Sector of the American Expeditionary Forces at Dijon, France, comprising waters from both treated and raw supplies and from various sources. An interesting correlation is noted between the rate of gas production and the probability of confirmation. It appears evident that

1. The positive presumptive test (10 percent or more gas in 24 hours) is a very reliable index of the probable presence of *Bact. coli*

when dealing with untreated waters but it is not to depended upon when testing chlorinated waters; 97.7 per cent of the former and only 44 per cent of the chlorinated samples showing gas, were confirmed for *Bact. coli* or its close allies.

2. The doubtful presumptive test (less than 10 percent gas in 24 hours, or more than this quantity in 48 hours where 24 hours was negative) is only a fair index of the probable presence of *Bact. coli* in untreated waters, while for chlorinated specimens it is practically negligible, as 75.9 percent of gas tubes from untreated, and only 7.6 percent of those from chlorinated samples were successfully confirmed.

3. A small amount of gas in 24 hours is a more reliable index for *Bact. coli* than 10 percent or more gas in 48 hours. The rate of gas production seeems more significant than the total volume of gas formed as a presumptive test.

Similar results were obtained by Graff and Mote. Of 966 instances of gas production in lactose peptone bile from settling basins (unchlorinated) 41 percent were positive. Of 844 instances from reservoirs (after chlorination) 12 percent were confirmed, and 1063 gas tubes from taps only 5 percent were confirmed. They also noted that vigorous gas production in 24 hours yielded positive tests for *Bact. coli* in a greater percent of cases, from all sources, than was obtained if gas was not noted previously to the 48 hour period.

It is thus apparent that gas production in either lactose broth or lactose bile, particularly with treated, partially purified, or chlorinated waters, cannot be relied upon as an index of the presence of the colon group. Confirmation must be restored to, except possibly for water known to be polluted.

CONFIRMATORY TESTS

it is merely necessary to streak out or smear on the surface of a differential medium (litmus lactose agar, Endo agar, or Eosin-Methylene-Blue agar) which is then included at 37° C. for 24 hours. If typical colonies develop, the presence of the colon group in the presumptive test is considered confirmed. This is known as the **partially confirmed test** and for routine work is sufficient, although it must be borne in mind that there are a few spore forming lactose fermenters which are capable of growing aerobically.

If the colonies developing on this differential medium are not typical it becomes necessary to further study these colonies to determine that they are Gram negative non-spore formers and that they are capable of producing gas from lactose. The latter may be determined by merely fishing a colony into lactose broth which is incubated for 24 hours. If gas is formed and if the Gram stain showed the colony to be a non-spore forming Gram negative short rod, the test constitutes what is spoken of in the Standard Methods as the **completed** test for the colon group.

If no growth at all develops on the confirmatory differential medium the assumption is that the gas in the presumptive test tube was due to strict anaerobes and the colon group is recorded as absent. It is con-

ceivable, of course, that such a test may be due to death of the colon types. A transfer from this unconfirmed presumptive test to another tube of lactose broth, which on incubation shows gas production, would be excellent evidence that the gas forming organism in question was an anaerobe and that the negative confirmatory test was not due to death or overgrowth of the gas producer.

The media for confirmatory tests which have found most favor among American bacteriologists are litmus lactose agar, and Endo agar; and more recently the simplified Eosin-Methylene-Blue agar was suggested by the author. The litmus lactose agar has been discussed in detail in connection with the isolation of the colon group. It will merely be added here that Meyer suggests that by the addition of 3 percent agar, giving a stiffer and dryer medium, more favorable results are obtained than with the ordinary medium.

The Endo Agar. This is perhaps the most extensively employed confirmatory medium at the present time. It is the medium of choice of the Public Health Service and is recommended in the Standard Methods (1917 and 1920). The colon colonies produce a distinct red coloration, often with a metallic sheen, and are quite easy to detect. Unfortunately there has been, in the past, considerable lack of uniformity in the preparation of Endo. The method of adjustment of the reaction is very crude and there was lack of agreement as to the quantity of indicator to be employed. Thus Kendall, and Prescott and Winslow recommended 0.1 c. c. of a saturated basic fuchsin and 1 c. c. of a 10 percent sodium sulphite per 100 c. c. of agar. The Army Laboratory Manual, employed extensively during the war, recommends 0.18 c. c. of fuchsin and 2.5 c. c. of a 10 percent sodium sulphite. In the Hygienic Laboratory Endo medium the indicator consists of 0.5 c. c. of basic fuchsin and the equivalent of 2.5 c. c. of a 10 percent sodium sulphite solution. Some investigators, Robinson and Rettger, have recommended decolorization with sodium acid sulphite in place of sodium sulphite.

The appearance of the colon colonies will depend to a considerable extent on the concentration of the indicator, being very intense red and showing the characteristic metallic sheen only with the higher concentrations. The description of a typical colony of *Bact. coli* on one of these mediums may therefore not tally at all with its appearance on supposedly the same medium prepared in some other laboratory. It thus becomes essential to detail, in each instance, the method of preparation of the medium employed. We may well exclaim with Morse and Wolman, "What shall the standard test for *Bact. coli* include if a mere difference in the proportion of fuchsin and sulphite in two Endo media results in the one showing typical colonies in 75 percent of the tubes, in which *Bact. coli* is present, while the other shows the same colonies in only 14 percent?" The apparent difference is due to a confusion of the term "typical" *Bact. coli* colony. What is considered typical for one medium with one concentration of dye will of course not be typical for another.

One of the great difficulties in the preparation of Endo medium is the adjustment of the reaction. In most of the methods the reaction is adjusted on the phenolphthalein scale to some definite point and then a quantity of sodium carbonate is added to make it more alkaline. This is a rather inaccurate method and results in some batches being very serviceable while others are almost worthless.

Levine suggested a modified and simplified Endo medium which requires no adjustment of reaction and which need not be filtered. The medium consists of 1 percent Difco peptone, 0.3 percent dipotassium phosphate, 0.5 percent agar, and 1 percent lactose. One-half c. c. of a saturated basic fuchsin, decolorized by 2.5 c. c. of a 10 percent sodium sulphite, as recommended by the Hygienic Laboratory, is employed as an indicator for each 100 c. c. of the medium. Aside from the simplicity of preparation, an advantage claimed is that *Bact. coli* may be differentiated from *Bact. aerogenes.* The former possess a distinct metallic sheen, the colonies are flat and button-like, and about two or three m. m. in diameter; whereas the latter usually produces considerably larger colonies which are convex and a metallic sheen is rarely observed. The disadvantage of the medium is that diffusion of color, due to acid production, is very rapid. This may be reduced by increasing the content of agar but when that is done the differentiation between *Bact. coli* and *Bact. aerogenes* becomes less distinct.

All of the Endo mediums above have the disadvantage of instability. Exposure to light or air induces a deep red coloration which interferes seriously with the detection of acid formers thus making it necessary to prepare the medium fresh and at frequent intervals.

Kahn suggests the use of Endo medium in tubes instead of plates inoculating from the presumtive test onto the slant and also into the butt in a manner analagous to the Russel double sugar medium. He states that Endo agar prepared and sterilized in tubes may be kept from three to four weeks without deterioration and that anaerobes will not develop in the butt of the Endo agar tube. Thus the test for acid production under aerobic and gas formation under anaerobic conditions by members of the colon group is determined simultaneously thereby performing both partially confirmed and a complete test, described above in a single tube. This use of the Endo medium is very interesting and suggestive and worth trial and consideration.

The Eosin Methylene Blue Agar Confirmatory Medium. Levine, in 1917, suggested a modification of the eosin-methylene blue agar (first described by Holt, Harris and Teague) for the confirmation of the presumptive test. The medium is prepared in the following manner:

Distilled water....................................1000 c. c.
Peptone (Difco)......................................10 gm.
Dipotassium phosphate..............................2 gm.
Agar...15 gm.

Boil ingredients until dissolved and make up any loss due to evaporation. Place measured quantities in flasks and sterilize at 15 pounds for 15 minutes.

Just prior to use, add to each 100 c. c. of the melted agar, prepared as above, the following constituents:

Sterile (20%) lactose solution............1 gm. or 5 c. c.
Aqueous (2.0%) eosin (yellowish) solution....2 c. c.
Aqueous (0.5%) methylene blue solution......2 c. c.

Pour medium into petri dishes, allow it to harden in incubator and inoculate in the ordinary way. Smearing the surface with a glass rod seems preferable to the streaking method sometimes employed.

There is no adjustment of reaction and filtration of medium is not necessary.

Test tubes may be substituted for petri dishes if desired. The value of such a change is (1) the reduction of expense, as only 3 or 4 c. c. of medium is needed for every test tube while about 15 c. c. is usually employed with petri dish, and (2) test tubes may be stored for long periods whereas the medium in petri dishes would have to be prepared at intervals of a week or less.

TABLE XXVII. DIFFERENTIATION OF *BACT. COLI* AND *BACT. AEROGENES* ON EOSIN-METHYLENE BLUE AGAR.

	Bact. coli (1)	*Bact. aerogenes* (2)
Size	Well isolated colonies are 2—3 m.m. in diameter.	Well isolated colonies are larger than coli; usually 4-6 m.m. in diameter or more.
Confluence	Neighboring colonies show little tendency to run together.	Neighboring colonies (2) run together quickly.
Elevation	Colonies slightly raised; surface flat or slightly concave, rarely convex.	Colonies considerably raised and markedly convex; occasionally the center drops precipitately.
Appearance by transmitted light	Dark almost black centers which extend more than ¾ across the diameter of colony; internal structure of central dark portion difficult to discern.	Centers deep brown; not as dark as *Bact. coli* and smaller in proportion to the rest of the colony. Striated internal structure often observed in young colonies.
Appearance by reflected light	Colonies dark, button-like, often concentrically ringed with a greenish metallic sheen.	Much lighter than *Bact. coli.* Metallic sheen not observed except occasionally in depressed center when such is present.

(1) Two other typea have been occasionally encountered: One resembles the type described, except that there is no metallic sheen, the colonies being wine colored. The other type of colony is somewhat larger (4 m. m.) grows effusly. and has a marked crenated or irregular edge, the central portion showing a very distinct metallic sheen. These two varieties conatitute about 2 or 3 percent of the colonies observed.

(2) A small type of aerogenes colony about the size of the colon colonies, which showa no tendency to coalesce, has been occasionally encountered.

The advantages claimed for the eosine-methylene-blue medium are (1) ease of preparation, as neither filtration nor adjustment of the reaction is required, and (2) relative permanency. (Petri dishes poured have been used after a week or longer, if stored in the ice box) and (3) the medium affords an excellent differentiation between *Bact. coli* and *Bact. aerogenes*, as described in Table XXVII above.

The method of preparation of the test tubes is briefly as follows:

The medium prepared as above is poured aseptically into sterile test tubes and allowed to solidify so as to give a long slant without a butt. To confirm the presumptive test, inoculation is made on the surface of these slants with a straight platinum needle. If desired the medium may be placed in test tubes and sterilized in the Arnold or in the auoctlave in the ordinary manner. In that case it will be found that the agar, when first removed from the sterilizer, is fluorescent like eosin but as it cools the typical wine color returns.

The reliability of the differentiation of *Bact. coli* and *Bact. aerogenes* is indicated in the following table:

TABLE XXVIII. RELIABILITY OF PRESUMPTVE DIFFERENTIATION OF *BACT. COLI* AND *BACT. AEROGENES* ON EOSIN-METHYLENE BLUE AGAR.

Designation from appearance on agar	1918-1919, France		1917, Iowa	
	Number of colonies tested	Percentage correct	Number of colonies tested	Percentage correct
Bact. coli	87	94.2	102	96.9
Bact. ærogenes	55	85.5	122	82.4

Thus in 1917, of 122 colonies fished, as probably *Bact. coli*, 96.9 percent were proved to be such and in 1918 and 1919, 94.2 percent of 87 colonies fished were correctly designated from their appearance on eosin-methylene blue agar. The proportion of *Bact. aerogenes*, correctly indentified was 82.4 percent and 85.5 percent in the series in Iowa and France respectively. The medium therefore appears to be quite reliable for the routine presumptive differentiation of these two groups of colon bacteria and may therefore be of considerable value if in the future it is considered desirable to distinguish these forms because of their probable different sanitary significance.

V. THE COLON GROUP AS AN INDEX OF POLLUTION

Safe Water. A safe water for human consumption may be defined as one which is free from harmful constituents important among which are disease producing microorganisms. The logical and most direct procedure to determine the potability and safety of a water would be to determine the presence or absence of pathogenic bacteria but unfortunately this task is an impossible one for routine and recourse must therefore be taken to an indirect index of the probable presence of harmful germs. Since the diseases transmissable through water are primarily of intestinal origin the detection of the presence of intestinal material naturally leads to the presumption that a potential danger exists, for if such material is present it is very probable and certainly possible that intestinal disease germs are also present.

A number of tests both chemical and bacteriological have been suggested as indicators of intestinal pollution. The bacterial examination, by reason of the large number of bacteria present in feces and sewage and the ease with which they may be detected in water, is a particularly delicate test. Three groups of bacteria have been regarded as indicators of pollution:

The colon group.
Sewage streptococci.
Spore forming anaerobes.

An organism to be considered an ideal index of fecal pollution should have the following characteristics:

1. It should be distinctively and characteristically of human or animal intestinal origin.
2. It should be absent or extremely rare in nature outside of the intestinal tract.
3. It must be capable of easy and rapid detection.
4. Its incidence in water should bear some constant relation to the sanitary survey or our knowledge as to the probability of pollution, particularly with sewage.
5. It should be distinctly more viable and more resistant in water and to treatment than are the intestinal pathogens (*Bact. typhi, Bact. dysenteriae,* etc.), but not excessively so.

Such an ideal index is not available but the general concensus of opinion among English and American bacteriologists is favorable to the employment of the Colon group for this purpose. Although bacteria of this group are not restricted in habitat to the intestinal tract of man being characteristic also of the intestinal tract of the lower animals, it is nevertheless true that there is a correlation between the quantitative incidence of a least the coli section and known pollution. The whole group is easy of detection as will be seen from the following considerations. It is more viable than *Bact. typhi* but yet dies off relatively quickly; colon bacilli are present in relatively large numbers in water known to be polluted but only infrequently in natural supplies. A correlation has been established

between the incidence of the colon group in drinking water and the typhoid fever rate in the community.

Viability of *Bact. coli* and *Bact. typhi* in Water. These organisms do not find favorable conditions for growth in natural waters. The food supply is apparently insufficient or unavailable, the temperature is unfavorable, other organisms exert a deleterious effect, and in consequence a diminution in number rapidly ensues. It is conceivable that under certain conditions of extremely high temperature, as may occur in summer, there may be an initial increase but death soon results. A convenient method for appraising the relative viability of two organisms is to compare the rates at which they die off under a given set of conditions.

The rate of death of bacteria may be expressed by

$$K = \frac{1}{T_1 - T_0} \log \frac{N}{n} \text{ where,}$$

"N" is the number of organisms at the beginning, i. e. time "To".

"n" is the number of organisms at the end of the time, "T_1".

"K" is a measure of the rate of death.

If the logarithms of the number of surviving bacteria at different periods are plotted against the elapsed time it will be observed that the points fall along a straight line intersecting the "x" axis at an angle whose tangent is numerically equivalent to "K". The greater the rate of death the greater will be "K". In the following tables are given the number of surviving *Bact. coli* and *Bact. typhi* when stored in water and exposed to the air. It will be observed that the rate of death of *Bact. typhi* is considerably higher than *Bact. coli,* though the latter organism also dies off rather rapidly.

TABLE XXIX. VIABILITY OF *BACT. COLI* IN WATER AT 20° C.
(After Hinds 1916)

Elapsed time in hours	Count	Rate of death "K"
0	690,000	
2	600,000	.069
6	420,000	.083
13	244,000	.079
24	123,000	.071
36	10,000	.1181
48	3,400	.111
72	120	.120

Average "K" 0.094

TABLE XXX. VIABILITY OF *BACT. TYPHI* IN WATER AT 20° C.
(After Hinds 1916)

Elapsed time in hours	Count	Rate of death "K"
0	118,000	1.38
2	7,500	1.38
4	500	1.37
6	280	1.01
8	230	.78
10	150	.67
12	30	.69
14	30	.59
24	2	.46

Average "K" 0.87

The writer made some observations at the Massachusetts Institute of Technology in 1913 on the effect of temperature on the viability of *Bact. coli* in distilled and conductivity water containing C. P. salt. The results are shown in figures 3 and 4 where it is apparent that the higher the temperature the greater the rate of death, and that the presence of gases in solution also affects the death rate.

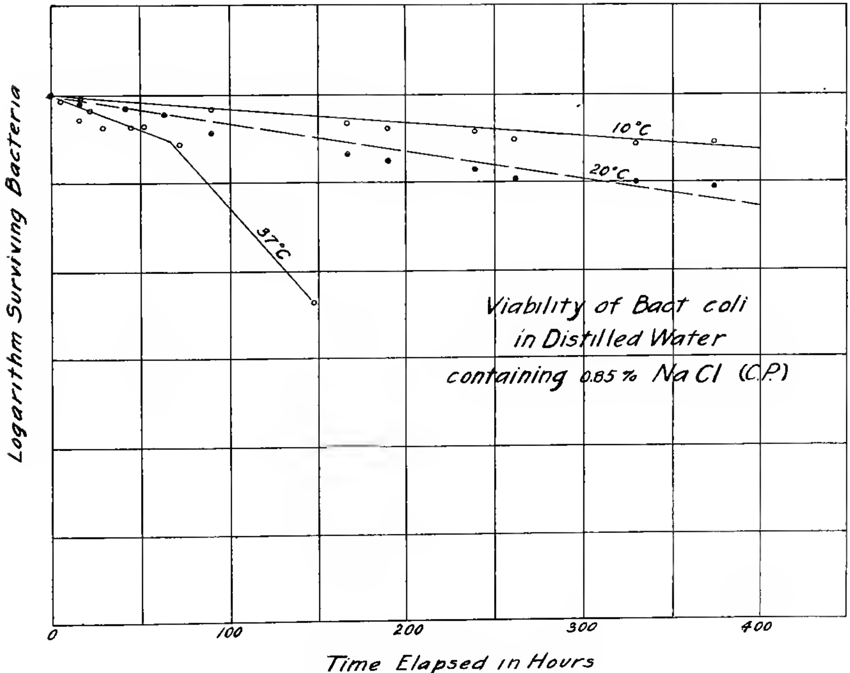
Houston artificially infected Thames River water with pure cultures of *Bact. typhi* and noted the proportion of survivors are infleuced by the temperature of storage. The results, based on ten experiments at each temperature, are sunimarized below:

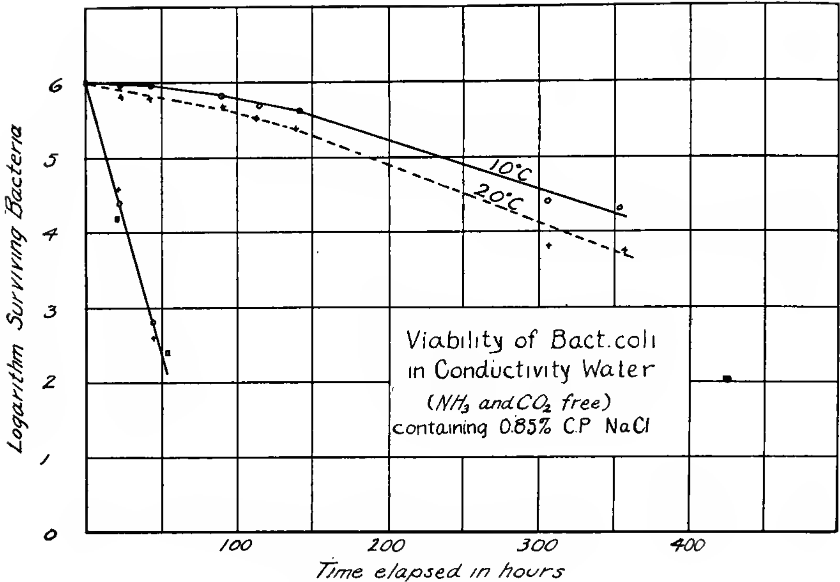
TABLE XXXI. EFFECT OF TEMPERATURE ON VIABILITY OF *BACT. TYPHI* IN THAMES RIVER.

(After Houston 1913)

Temperature of storage	Surviving *Bact. typhi* per c. c. after 1 week*	*Bact. typhi* absent from 1 c. c. after
0° C.	47,766	9 weeks
5° C.	14,894	7 weeks
10° C.	69	5 weeks
18° C.	39	4 weeks
27° C.	19	3 weeks
37° C.	5	2 weeks

*Initial number 103,328 per c. c.





That there is a marked correlation between the incidence of the colon group and the probability of pollution may be clearly demonstrated from Houston's observations on brooks and rivers which are more or less subject to pollution, and on two lakes, one of which, Loch Loggan, was receiving some drainage while the other, Loch Ericht, was free from contamination of human or domestic animals. It will be observed that in brooks and rivers, colon bacilli were usually present (61.5%) in small portions of water (1 c. c. or less); whereas in the unpolluted, Loch Ericht, the colon group was only occasionally encountered even in 100 c. c. samples.

TABLE XXXII. ON THE INCIDENCE OF COLON GROUP AND PROBABILITY OF POLLUTION IN SURFACE WATERS.
(After Houston 1906)

Source	Contamination	% of Samples showing *Bact. coli* in				
		+0.1 c. c.	+1.0 c. c. —0.1 c. c.	+10 c. c. —1.0 c. c.	+100 c.c. —10 c.c.	Absent from 100 c. c.
Brooks & rivers	More or less polluted	7.7	53.8	34.6	3.8	
Loch Loggan	Receives some farm land drainage		1.2	33.0	49.4	16.4
Loch Ericht	Free from human & animal pollution			1.0	19.0	80.0

Cumming in his report on the sanitary conditions of the Potomac River watershed found a striking correlation between the proximity of pollution and the incidence of colon bacilli.

TABLE XXXIII. ON THE RELATION OF THE NUMBER OF COLON BACILLI AND PROXIMITY OF POLLUTION IN SURFACE WATER (POTOMAC RIVER).

(After Cumming 1916)

Sampling Point	Distance from source of pollution	Bacilli of colon group per c. c. monthly average
Just below city		24 to 617
At Marvel Hall	14 miles	24 to 137
Maryland Point	42 miles	.04 to 6.9
Oyster region	64 to 103 miles	.01 to .04

The high incidence of the colon group in the vicinity of sewer outlets, its elimination as the distance from the source of pollution increases, and its rarity at points remote from contamination is clearly shown.

In connection with the purification of water, some correlation has been observed between the incidence of colon bacilli in the filtered water and the typhoid fever rate in the community. Thus in Lawrence, Massachusetts, Clark and Gage report the following incident, which occurred in 1898. In relaying some under drains of a filter the sand on the beds was seriously disturbed resulting in a marked increase in tests for colon bacilli in the filter effluent. This was followed by a rapid rise in the number of cases of typhoid fever, and as the colon content fell to normal, the cases of typhoid fever disappeared.

TABLE XXXIV. SHOWING RELATION OF COLON INCIDENCE TO TYPHIOD MORBIDITY RATE, LAWRENCE, MASS., 1898-9.

(After Prescott & Winslow, 1915)

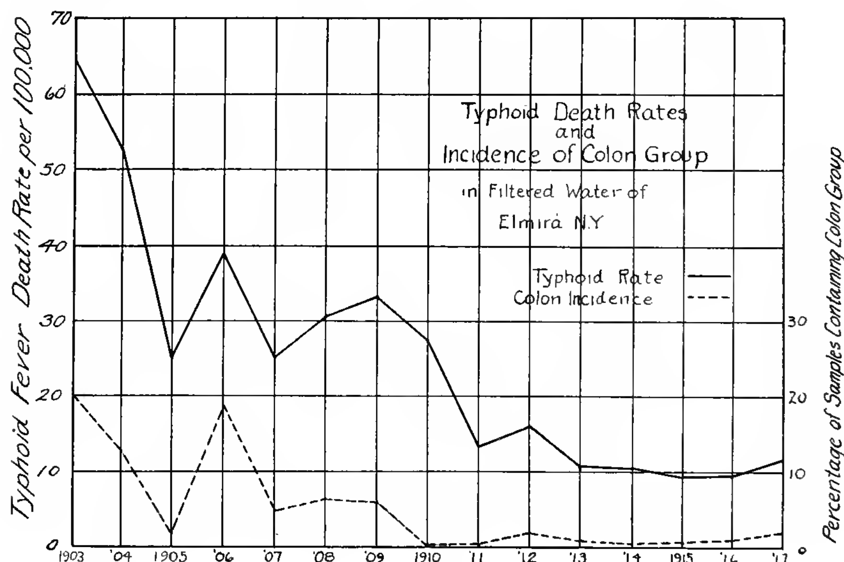
	Percent of positive colon tests in 1 c. c. samples	Number of cases of typhoid fever
December, 1898	72	12
January, 1899	54	59
February, 1899	62	12
March, 1899	8	9 (all during the early part of the month)

Caird, in a report on twenty years' experience with the filter plant at Elmira, New York, also notes that there is some correlation between the percent of positive colon tests and the typhoid fever rate.

Opinions as to the Value of the Colon Test. It appears, from the foregoing discussion, that the colon group possesses many of the attributes of an excellent indicator of pollution. Unfortunately, however, it is not characteristic and distinctive of the human intestine. Some of its members have been isolated from grain and other sources which are far removed from human or even animal contamination. This has lead to disagreement as to the value of the colon test in water analysis.

TABLE XXXV. INCIDENCE OF COLON GROUP AND TYPHOID DEATH RATE IN FILTERED WATER, ELMIRA, N. Y. (After Caird 1919).

Year	% Samples showing colon group	Typhoid rate for 100,000
1903	20.00	64.5
1904	13.33	53.3
1905	1.37	25.2
1906	18.71	39.2
1907	4.17	25.2
1900	6.04	30.8
1909	5.47	33.6
1910	0.00	26.4
1911	0.00	13.2
1912	1.81	15.9
1913	0.46	10.6
1914	0.00	10.6
1915	0.17	9.6
1916	0.35	9.6
1917	1.43	11.9



The English and American bacteriologists uphold it. The Germans have objected strenuously to its inclusion in water work. The contention of Kruse, Konrich and other German investigators that colon bacilli may be found in any water irrespective of its source, provided a sufficiently large sample is taken, and that of Chick, Houston, and other English authorities, that these organisms are not present in water unless it has received

sewage pollution, led Prescott and Winslow to conclude that it was the **number** rather that the **presence** of colon bacilli that should be used as a criterion for recent sewage pollution. Thus they state that the finding of a few colon bacilli in large samples of water or their occasional isolation from small samples is not of any special significance but if these organisms are present in a large proporotion of small samples (I c. c. or less) then the evidence of recent sewage pollution is significant.

Gärtner (1910) estimates the number of colon forms in cultivated soil at 1,500,000 per square meter and naively remarks that it is no wonder the rain should wash a few of them into neighboring wells. The recent work of Johnson and Levine, Burton and Rettger, Chen and Rettger and others have shown quite conclusively that the organisms in the soil are markedly different from those habitually present in the human and animal intestine. Furthermore the "*B. coli*" of Houston and other English investigators does not include many of the soil forms for they regard only lactose positive-indol positive varieties as "typical" *B. coli*, whereas most of the *Bact. aerogenes* and *Bact. cloacae* are indol negative. German opinion is gradually becoming more favorable toward acceptance of the colon test, provided that preliminary enrichment is carried out at 46° C. (Eijkman test). This procedure, as has been pointed out before, serves to eliminate the *Bact. aerogenes* and *Bact. cloacae* or characteristic soil types. It appears that the contentions of the German school were really against these soil forms as indices of pollution. It thus becomes possible to reconcile their opinions with those of English and American bacteriologists if we recognize that not only is the numerical incidence of the colon group significant as an index of pollution, but that the type of organism must also be considered.

THE COLI AND AEROGENES SECTIONS AS INDICES OF POLLUTION

The question arises as to whether the aerogenes and coli sections are to be accorded the same sanitary significance. Considerable work is still needed along these lines, but the following is presented as to (1) their distribution in nature, (2) correlation of their relative incidence with the sanitary qualities of water, (3) relative viability in water, and (4) relative resistance to treatment and chlorination.

Incidence of the Coli and Aerogenes Sections in Feces. Rogers, Clark, and Evans called attention to the scarcity of the high ratio group (aerogenes section) in cow dung. Only one colony of 150 fished was of this type. Levine observed that among 117 cultures isolated from cow, horse, sheep, pig, and man, not a single organism proved to be *Bact. aerogenes*. A study of the literature employing the Voges-Proskauer test as a means of differentiation of the two colon sections disclosed that these observations as to the scarcity of the *aerogenes* types in human and animal feces has long been recognized although not adequately appreciated.

Thus MacConkey (1905) remarks on the scarcity of *Bact.* (*lactis*) *aerogenes* in human feces. In the examination of 241 strains obtained from

23 human stools, only 4 gave the Voges-Proskauer reaction and of these 3 were obtained from a single sample. At the same time, a study of 51 strains from 5 samples of horses and 48 from 6 samples of cow manure did not yield a single aerogenes culture. In 1909 he made further observations with similar results.

Ferriera, Horta and Paredes reported 8 out of 117 lactose fermenting strains from human feces, and 2 of 81 strains obtained from 46 different species of animals to belong to the aerogenes section as indicated by a positive Proskauer test.

The work of Clemesha is particularly significant because of the large number of cultures examined. Of 1207 human strains, about 6 percent were found to be of the aerogenes type but a large number of these came from a single sample. Among 1029 cultures from cow dung, *Bact.* (*lactis*) *aerogenes* was encountered in about 10 percent. He records that in cow dung, the *Bact. aerogenes* was found in small numbers, and *Bact. cloacae* was sometimes very common, but that in human stools these types were very rare and that a sudden increase in their prevalence was never observed. Clemesha's observations were confirmed to a considerable extent by R. G. Archibald of the Wellcome Tropical Research Laboratory in an investigation of the water of Khartoum.

TABLE XXXVI. INCIDENCE OF AEROGENES TYPES AMONG COLON BACILLI IN HUMAN DEJECTA.

Investigators	No. of strains studied	% Aerogenes Section (V. P.+ :M. R.—)
MacConkey (1905)	241	1.7
Ferriera, Horta and Paredes (1908)	178	5.6
MacConkey (1909)	117	6.8
Archibald (1911) *	100	0.0
Clemesha (1912)	1207	4.6
Levine (1916)	25	0.0
Hulton (1916)	10	0.0
Rogers. Clark and Lubs (1916)	113	5.8
Rogers, Clark and Lubs (1918)	177	†23.0
Darling (1919)	20	0.0
Wood (1919)	33	0.0
Stokes (1919)	141	§16.3
Chen and Rettger (1920) ‡	173	0.0
Total	**2534**	**5.9**

*Includes strains obtained from latrines, directly from feces, and fecal suspensions exposed to action of sunlight.

†These comprised 46 strains isolated for the most part by special methods and 31 of these strains were obtained from a single specimen. The true incidence of aerogenes types is therefore considerably less than shown in the table.

§All **Bact. cloacae.**

‡Includes animal strains.

Recent observations by Rogers and his associates, Darling, Chen and Rettger, Stokes, and others are entirely in accord with the opinions recorded that the aerogenes section is rare in feces of man and animals.

The relative presence of the coli and the aerogenes sections in human and various animal feces is shown in Tables XXXVI. and XXXVII.

TABLE XXXVII. INCIDENCE OF AEROGENES TYPES AMONG COLON BACILLI IN ANIMAL DEJECTA.

Investigators	Animal source	No. of strains studied	% Aerogenes Section (V.P.+; M.R—)
MacConkey (1905)	Horse & cow	99	1.0
Ferriera, Horta & Paredes (1908)	Misc.	81	2.5
MacConkey (1909)	Horse	67	4.5
MacConkey (1909)	Misc.	87	0.0
Archibald (1911)	Cow & goat*	20	40.0
Clemesha (1912)	Cow	1029	10.7
Rogers, Clark & Evans (1914)	Cow	150	0.7
Levine (1916)	Misc.	92	0.0
Darling (1919)	Misc.	93	0.0
Wood (1919)	Misc.	99	7.0
Stokes (1919)		15	13.0
Total		**1832**	**7.4**

*Single samples from each species.

Incidence of the Coli and Aerogenes Sections on Grains and in Soil. Many investigators have reported the presence of the colon group on grains and plants. Prescott found lactose fermenting bacteria in flour, bran, corn meal, oats, and barley. Similar observations were made by Papasotiriu in Germany. Cline and Houston found, what they regarded typical colon baccili, in 3 out of 24 samples of wheat, oats, rice, etc. Bettencourt and Borges (1908) succeeded in isolating such organisms from vegetables and cereals. Konrich (1910) frequently observed the colon group on cultivated plants and that even about six percent of plants obtained from waste places showed colon bacilli. Fifty-five percent of 300 samples of grain showed members of the colon group.

These investigators, however, did not differentiate between the *Bact. coli* and *Bact. aerogenes.* Rogers, Clark, and Evans were the first to point out that the colon-like forms, occurring on grains, were distinctly different from those observed in feces. They isolated 166 cultures from 33 samples of dried grains and 2 samples of green oats. Of these only 8 (4.8%) were the low ratio (coli section); (91.0%) were of the

high ratio type (aerogenes section), and 7 (4.2%) produced only hydrogen. They noted further that none of the types found on grains were identical with those obtained by them from bovine feces, the low ratio types from grains being pigmented.

Stokes isolated 77 strains from corn meal, grape nuts, post toasties, corn flakes, puffed wheat, quaker oats, rye, corn, wheat, barley, and oats, of which 57 (74.0%) were of the aerogenes cloacae type. It is apparent therefore that on grains and plant products, the high ratio V. P. positive, methyl-red negative group predominates.

Houston, in 1897 and 1898, examined a number of samples of orchard, garden pasture, and virgin soil, including polluted and unpolluted areas, concludes that the true colon bacilli are very rarely or never found in virgin soils, while they are present in large numbers in other areas, especially those which have been contaminated from animal sources.

Konrich, in a study of 547 specimens of soils in Germany, detected the colon group in 65 percent of 0.1 to 0.5 gram samples. He notes that

TABLE XXXVIII. SOURCE, TREATMENT, ETC., OF 42 SAMPLES OF SOIL EXAMINED IN 1915.

Sample	Source	Soil treatment or other remarks	Date of last treatment	Crop	Number of coli-like organisms obtained
A	Corn field	Manured, corn grown for three years	1913	Corn	0
B	Corn field	Manured, corn grown for three years	1913	Corn	14
C	Corn field	Manured, corn grown for three years	1913	Corn	4
D	Corn field	Manured, corn grown for three years	1913	Corn	3
E	Corn field	Manured, corn grown for three years	1913	Corn	2
H	Corn field	Manured, corn grown for three years	1913	Corn	9
I	Expt. plot 114	4 tons manure annually	1914	None	0
II	Expt. plot 113	2 tons manure annually	1914	None	0
III	Expt. plot 112	1 ton manure annually	1914	None	0
IV	Expt. plot 111	4 tons of clover chopped and ploughed under annually	1914	None	0
V	Expt. plot 110	2 tons of clover chopped and ploughed under annually	1914	None	18
VI	Expt. plot 109	1 ton of clover as in IV	1914	None	0
VII	Expt. plot 108	2 tons of oat straw chopped and ploughed under annually		None	0
VIII	Expt. plot 107	No treatment, check plot	1914	None	1
IX	Expt. plot 106	2 tons of timothy chopped and ploughed under annually	1914	None	0
X	Expt. plot 105	1 ton timothy as in IX	1914	None	1
XI	Expt. plot 104	8 tons of clover once in four years	1913	None	26
XII	Expt. plot 103	8 tons of manure once in four years	1913	None	24
XIII	Expt. plot 102	28 tons peat annually	1914	None	14
XIV	Expt. plot 101	Timothy grown each year and stubble plowed under	1914	Timothy	18

TABLE XXXVIII. (continued)

Sample	Source	Soil treatment or other remarks	Date of last treatment	Crop	Number of coli-like organisms obtained
XV	Orchard	Clover sod		Clover	12
XVI	Orchard	Clover crop planted in summer and ploughed under in spring		?	0
XVII	Orchard	Clean cultivation			0
XVIII	Orchard	Blue grass sod		Blue grass	8
XIX	Sioux loam	Clover field recently in oats		Clover	1
XX	Wabash silty clay	Surface soil			1
XXI	Marshal silt loam	Surface soil			8
XXII	Corrington loam	Surface soil; corn field		Corn	5
XXIII	Expt. plot 213	No treatment, check plot		Corn	13
XXIV	Expt. plot 214	Legume treatment, cow peas in July. 2 years out of four in rotation with corn	1913	Corn	18
XXV	Expt. plot 215	8 tons manure	1913	Corn	15
XXVI	Expt. plot 216	8 tons manure with cow peas	1913	Corn	14
XXVII	Expt. plot 217	8 tons manure with 800 pounds bone meal and cow peas	1913	Corn	16
XXVIII	Expt. plot 218	800 pounds bone meal and cow peas	1913	Corn	16
XXIX	Expt. plot 219	8 tons manure and 800 pounds bone meal	1913	Corn	13
XXX	Expt. plot 220	800 pounds bone meal and 200 pounds K in KCl and cow peas	1913	Corn	19
XXXI	Expt. plot 221	8 tons manure, 800 pounds bone meal and 200 pounds K in KCl	1913	Corn	12
XXXII	See XIV	See XIV	1913	Timothy	10
XXXIII	Clover field	Unknown	1913	Clover	13
XXXIV	Corn field	Same as A	1913	Corn	17
XXXV	Corn field	Same as A	1913	Corn	15
XXXVI	Corn field	Unknown	1913	Kafir corn	2

the farther away the source was from cultivation, the smaller was the proportion of positive results, but that they were never altogether absent. Unfortunately, neither Houston nor Konrich differentiated between the aerogenes and coli sections.

Johnson and Levine first pointed out that the coli-like microorganisms of the soil were strikinglv similar to those obtained by Rogers and his associates from grains and that they were quite distinct from the true *Bact. coli* of feces.

The observations of Johnson and Levine are based upon 42 samples of soil obtained from experimental plots, (data on treatment of which is available for many years) from orchards, corn fields, and miscellaneous sources in different parts of the state of Iowa.

The method of isolation of cultures was by planting on litmus lactose agar directly or after preliminary enrichment in lactose broth, incubation

being at the body temperature. In the following table is given the source and treatment of the soil, crops raised, and the incidence of coli-like bacteria in the samples studied.

It will be noticed that a number of plots which had not received manure for many years (some about 15 years) yielded organisms of this group. There was a marked correlation also between cropping and the incidence of the colon group.

TABLE XXXIX. PRESENCE OF BACILLI OF COLON GROUP IN EXPERIMENTAL SOIL PLOTS AT AMES, IOWA

Coli-like organisms	Fallow plots		Cropped plots	
	Number	Percent	Number	Percent
Absent	7	53.8	0	0.0
Rare	2	15.4	0	0.0
Abundant	4	30.8	11	100.0

Of the 24 plots examined, 13 had been kept fallow and 11 had had various crops, for the most part corn, raised upon them. Of the fallow plots 7 (53.8%) showed no colon forms, in 2 (15.4%) such organisms were rare, and relatively abundant only in 4 (30.8%). On the other hand, bacilli of the colon group were readily isolated from each of the 11 plots upon which crops were grown.

One hundred and seventy-seven strains were obtained in pure culture. Of these 142 (80.4%) are of the aerogenes section (the predominating type being the *Bact. cloacae*) and 35 (19.6%) are of the coli section.

The preponderence of the aerogenes types in soil has recently been confirmed in two very detailed studies by Burton and Rettger (1917) and Chen and Rettger (1920).

Burton and Rettger isolated 193 coli-like bacteria from about 1000 samples of soil, twigs, leaves, and flowers.

Of these only 36 (18.7%) resembled *Bact. coli* whereas 157 (81.3%) were of the aerogenes subgroup, 148 of the latter being *Bact. cloacae.*

Chen and Rettger in a study of a large number of samples of soil of known sanitary quality observed also a great preponderence of the aerogenes section but they found the *Bact. aerogenes* rather than the *Bact. cloacae,* as recorded by Johnson and Levine and by Burton and Rettger, to be the predominating species. Of 467 strains studied, 430 (92.1%) were *Bact. aerogenes,* 17 (3.6%) *Bact. cloacae,* and only 20 (4.3%) were of the coli section (V. P. negative-methyl-red-positive type).

The evidence is quite distinct and clear that the colon-like organisms present in soil are of the aerogenes type and that they are more abundant in cropped than in fallow areas. The relative incidence of the aerogenes and coli sections in soil and grains is summarized in Table XL.

TABLE XL. INCIDENCE OF AEROGENES TYPES AMONG COLON BACILLI ISOLATED FROM SOIL AND GRAINS.

Investigator	Source	No. of strains studied	% Aerogenes section (V.P.+ M.R.—)
Levine & Johnson (1917)	Soil	177	80.4
Burton & Rettger (1917)	Soil	193	81.3
MacConkey (1909)	Soil	16	37.5
Chen & Rettger (1920)	Soil	467	95.7
Total . . .		**853**	**88.1**
MacConkey (1909)	Grains	30	56.7
Rogers, Clark & Evans (1915)*	Grains	166	91.0
Wood (1919)	Grains and Cereals	15	73.5
Stokes (1919)	Grains	77	74.0
Total . . .		**288**	**81.7**

*Differentiation based on gas ratio.

Incidence of the Coli and Aerogenes Sections in Water and Milk. There is, at present, comparatively little information available as to the relative abundance, of the coli and aerogenes subgroups in various types of water. Rogers, in a study of 137 strains isolated from a stream, found 66 percent were of the high ratio and aerogenes-cloacae and 33 percent of the low ratio coli type. Greenfield and Skorup found the ratio just the reverse. Among 405 colon strains, isolated from surface and ground waters in Kansas, 70 percent were of the low ratio V. P. negative type and 30 percent of the high ratio V. P. positive type. Houston, Winslow and Cohen, and Wood obtained similar findings to that of Greenfield. That is, they also found a preponderance of the coli subgroup in water; while Stokes, in a study of 528 cultures from water of Maryland, and MacConkey, among 49 strains from ponds, rain waters, and roof washings, obtained a preponderance of the so-called non-fecal or high ratio aerogenes and cloacae strains. Clemesha, in India, and Boles, in the Canal Zone, point out that the *Bact. coli* is in excess immediately after pollution but that later the *Bact. aerogenes* are the prevailing forms.

Although there is no agreement as to the relative incidence of these two groups in water, it is apparent that the aerogenes section is proportionately much more common than in feces and much less frequent than in soil. The author thinks it is a reasonable assumption that the strains in water represent the resultant of sewage pollution, soil contamination, and proximity of pollution.

The colon group isolated from milk is about evenly divided between aerogenes and coli types. Wood, MacConkey, Orr, Rogers and Stokes

recorded 17.5, 29, 39, 47, and 69.4 percent respectively of the aerogenes group in milk.

TABLE XLI. INCIDENCE OF AEROGENES TYPES AMONG COLON BACILLI ISOLATED FROM WATER AND MILK

Investigators	Source	No. of strains studied	% Aerogenes section (V.P.+;M.R.—)
Houston (1911)	Water	532	8.1
MacConkey (1909)	Water	49	59.2
Rogers (1916)	Water	137	66.0
Greenfield & Skorup (1917)	Water	405	30.0
Winslow & Cohen (1918)	Water	255	21.2
Wood (1919)	Water	231	33.3
Stokes (1919)	Water	528	63.7
Total . . .		**2137**	**35.2**
Orr* (1908)	Milk	850	39.0
MacConkey (1909)	Milk	26	57.8
Rogers, Clark & Davis (1914)	Milk	124	47.5
Hulton (1916)	Milk	18	72.3
Wood (1919)	Milk	93	17.5
Stokes (1919)	Milk	271	59.8
Total . . .		**1382**	**43.1**

*Glucose fermenters, lactose reaction not recorded.

The observations as to the incidence and distribution of the colon group in nature may be summed up as follows:

The evidence indicates that lactose fermenting bacteria capable of growing aerobically are widely distributed in nature and that they constitute two distinct groups; a low ratio V. P. negative subgroup, which was previously described as the coli section, is characteristic of the intestinal tract of man and animals and relatively infrequent in localities not recently polluted with human or animal intestinal material; and a high ratio V.-P. positive group, termed the aerogenes section, which is very rare in the intestinal discharges of man and animals, but constitutes the predominating type in soil and on grains. In water and milk the proportion of coli and aerogenes strains naturally varies, the predominating type depending to a considerable extent on the source of contamination.

Correlation of the Sanitary Survey and the Incidence of Coli and Aerogenes Types in Water. If, as has been intimated in the foregoing discussion, the coli and aerogenes sections are so characteristically

TABLE XLII. SUMMARY OF TYPES OF COLON BACILLI ISOLATED FROM VARIOUS SOURCES

Source	No. of strains observed	% Aerogenes section	% Coli section
Human feces	2534	5.9	94.1
Animal feces	1832	7.4	92.6
Water	2137	35.2	64.8
Milk	1382	43.1	56.9
Grains	288	81.7	18.3
Soil	853	88.1	11.9

of different origin, should we not expect a correlation of the sanitary survey with the type of colon bacillus encountered in a water? Reliable information on this matter would certainly be a great aid in the interpretation of water analyses. Winslow and Cohen studied the distribution of these organisms in waters of known sanitary quality with this idea in mind and concluded that there was no relation whatsoever between the incidence of aerogenes bacilli and the sanitary quality of the source. Thus among 94 strains isolated from polluted waters, 80 from unpolluted, and 81 from stored waters in the vicinity of New Haven, Connecticut, organisms giving the V.-P. reaction were encountered in 24, 24, and 15 percent respectively. They conclude that the "significance of the ratio of *Bact. coli* and *Bact. aerogenes groups* in a sanitary examination of water seems somewhat dubious." Their results are quite the reverse of those reported by other investigators.

Greenfield and Skorup conclude that there is a correlation between the increased incidence of the *Bact. coli* strains and the sanitary survey during dry weather.

TABLE XLIII. RELATIVE NUMBER OF AEROGENES AND COLI TYPES IN WOLF CREEK. (Rogers 1918)

Miles	Pollution	Cultures isolated	Ratio aerogenes to coli
0		7	All aerogenes
1.1	Private Sewers	15	1.1
2.1	City sewer	18	0.8
3.1		11	0.1
5.1		20	9.0
7.1		10	All aerogenes
9.1		9	3.5
11.3		5	4.0

Rogers studied a section of a stream for a distance of about eleven miles determining the ratio of *Bact. aerogenes* to *Bact. coli* above and below sewer outlets.

It will be noted that above the city all cultures isolated were of the aerogenes type and that after passing through the town, where the creek became polluted, the *Bact. coli* strains predominated, being 10 times as common, but farther down the stream, as the distance from the source of pollution increases, the aerogenes forms again become markedly more numerous. The indications are, therefore, strong that the ratio of aerogenes to coli varies with the proximity of sewage pollution becoming greater as the pollution is more remote.

Stokes remarks on the scarcity of the low ratio coli group in well waters. Only one low ratio organism was found in 14 samples of water from artesian wells containing members of the colon-aerogenes group. Thirteen of these contained high ratio organisms including *Bact. aerogenes* and *Bact. cloacae.*

In a study of 59 samples of raw waters, Stokes found 38 which showed only one type of the colon group (24 contained *Bact. aerogenes,* 13 *Bact. coli,* and 1 *Bact. cloacae*) determined by fishing 10 colonies from an Endo plate. This striking fact that a large proportion of the samples harbored a single species of the colon group in pure culture suggests the possibility of a correlation between the source of pollution or contamination and the type of colon organism present. He concludes that "In the bacteriological examination of drinking water it would not be safe to assume that the water was free from intestinal pollution if but two or three colonies were studied from each specimen. However, if 20 to 25 colonies be picked from each sample and all are found to be of the high ratio type, it might be safe to regard the water as free from fecal contamination provided a chemical examination and sanitary survey gave no evidence of pollution."

The writer would suggest that in place of picking such a large number of colonies, which is impractical for routine, a differential medium like the eosin-methylene-blue agar should be employed for confirming the presumptive test. The probable presence of the "fecal" *Bact. coli* type could be easily recognized.

Perhaps the most detailed study on the correlation of sanitary quality of ground water and the type of colon bacillus present was carried out by D. R. Wood in England. A large number of samples from various sources, 200 of which contained lactose fermenting organisms, were investigated. Sixty-six samples contained the V.-P. positive-methyl-red negative or *Bact. aerogenes* types and 41 of these showed no evidence of recent excretal contamination. He records also that in 22 deep well supplies of good repute (these were sunk through lime-stone 250 to 400 feet into underlying sand, used as public supplies for years, and appeared excellent bacterially, lactose fermenters being rarely present and seldom in less than 100 c. c.) suddenly showed a large proportion of the colon group in the spring of 1918. Lactose fermenting organisms were obtained in two cases in 1 c. c., in several instances in 10 c. c., but in every sample in 100 c. c. On further examina-

tion all but one of these organisms proved to be of the aerogenes section (V.-P. positive, methyl red negative). As these wells were several miles apart the simultaneous appearance of these organisms is difficult to explain. Wood mentions that the time of year rather suggests some connection with the sowing of grain.

It is felt that the relation of the sanitary survey to the type of colon bacilli present in the water, particularly well supplies, is in need of considerable investigation. The lack of correlation in the vicinity of the New Haven, reported by Winslow and Cohen, is difficult to reconcile with the observations of Stokes in Maryland and Wood in England. Possibly local conditions, if taken into proper consideration, may explain the seeming discrepancies.

On the Relative Viability of Bact. coli and Bact. aerogenes in Water. Emulsions of feces were stored in water in bottles or suspended in parchment sacks in running streams by Rogers. He reported that the *Bact. coli* died off much more rapidly than the *Bact. aerogenes.* In water kept in a bottle at 20° C., preliminary examination showed only *Bact. coli* but after 3 days storage the ratio of *Bact. aerogenes* to *Bact. coli* was 1 to 6. This ratio gradually increased until after 278 days storage the *Bact. aerogenes* was 39 times as prevalent as *Bact. coli.* This was of course a prolonged period of storage but it indicates, nevertheless, that the aerogenes group is more resistent and will persist for a much longer period in water than will the coli group.

In running water the change in the ratio of *Bact. aerogenes* and *Bact. coli* was much more rapid. Thus beginning with a fecal emulsion in parchment sacks suspended in running water, an initial ratio of 1 *Bact. aerogenes* to 2.3 *Bact. coli* was found after 7 days to contain 10 times as many *Bact. aerogenes* as *Bact. coli.* The *Bact. aerogenes* were proportionately more than 20 times as prevalent in a fecal suspension stored in running water for a week. The death rate of *Bact. coli* (K=0.48) was about twice that of *Bact. aerogenes* (K=0.93).

TABLE XLIV. CHANGES IN COLON BACTERIA IN RUNNING WATER.

(After Rogers 1918)

Age, days	Colon group, per c. c.	Ratio of *Bact. aerogenes* to *Bact. coli*
0	190,000	1 to 2.3
1	130,000	1 to 4.0
2	19,000	1 to 3.3
3	9,000	1 to 1.2
4	20	1 to 1.1
7	30	1 to 0.11

Winslow and Cohen report similar, though not such striking results, when storing mixtures of these organisms in bottles. They observed a

preliminary decrease in the proportion of *Eact. aerogenes*, after 24 hours, followed by an increase. An initial incidence of 46 percent *Bact. aerogenes* rose to 71 percent after 60 days' storage.

Savage and Wood, in studies on the vitality and viability of *Streptococci* in water also report a number of interesting observations on the relative incidence of the capsulated and non-capsulated members of the colon group as affected by storage. The following experiment is illustrative of their results.

To a liter of water which was not sterilized but which was known to be free from the colon group, were added 0.03 c. c. of a 24 hour peptone water culture of non-capsulated and capsulated colon organisms were added. The non-capsulated form (*Bact. coli*) with an initial count of 4,000 per c. c. fell to 25 per c. c., after 31 days, and 3 per c. c. after 33 days' storage and was entirely absent in 50 days. The capsulated form (*Bact. aerogenes*), on the other hand, was reduced from an initial count of 7,440 to 440 in 31 days, to 122 in 50 days, and living organisms were still present in this water at the end of 14 weeks. In another instance, an initial count of 8,000 non-capsulated organisms *(Bact. coli)* dropped to less than 1 per c. c., whereas the capsulated strain, with an initial count of 4,920, was still present to the extent of 540 per c. c. after 28 days' storage.

TABLE XLV. ON THE VIABILITY OF COLON GROUP IN UNSTERILE TAP WATER. (After Savage and Wood 1917).

Period of storage	Surviving bacteria per c. c.	
	Non capsulated (*Bact. coli*)	Capsulated (*Bact. aerogenes*)
At start	4,400	7,440
31 days	25	440
43 days	3	176
50 days	0	122
57 days	0	81
70 days		30
84 days		25
98 days		6

They also observed that when sewage was stored the *Bact. coli* strains gradually disappear the ultimate colon survivors being all capsulated strains (*Bact. aerogenes*).

Clemesha in India reports that *Bact. aerogenes* is relatively rare in waters recently polluted but becomes extremely common 5 to 15 days after contamination. He found the *Bact. aerogenes* more prevalent in rivers and lakes shortly after rains but was later supplanted by another member of the aerogenes section, *Bact. cloacae*, which was found to be the predominating type during dry seasons.

Similarly, Bowles, working with waters in the Panama Canal Zone, where the conditions simulate those of the tropics, notes that the aerogenes types are extremely prevalent long after the *Bact. coli* forms have died off. The supplies in the canal zone consist of impounded reservoirs and are treated with sulphate, filtered, and in some instances chlorinated after filtration. The reservoirs are policed and adequately protected against polluion. In the Chagres River during the rainy weather *Bact. coli* was present in 1 c. c. but during the dry season *Bact. coli* was found only in 50 c. c. samples while *Bact. aerogenes*, he says, was present in large numbers. During the dry season, the reservoirs, acting as sedimentation basins, effect considerable (wonderful) purification. He states that many examinations may be made this time of year without getting any test for *Bact. coli,* but that *Bact. aerogenes* is present.

There is no question, therefore, as to the relative viability of *Bact. coli* and *Bact. aerogenes* (and *Bact. cloacae*) in water. The latter is much more resistant, will persist for considerably greater periods, so that when present alone (that is, not accompanied by *Bact. coli*) in natural waters, it may merely indicate pollution or contamination so remote that the *Bact. coli,* and consequently the dangerous intestinal disease producing forms, have died off. In fact, Clemesha maintains that the presence of *Bact. aerogenes* is a favorable indication that self-purification is rapidly taking place.

Relative Resistance of Coli and Aerogenes Types to Chlorination and Filtration. Greenfield and Skorup state that there is no difference in the resistance of these two forms to treatment.

Ellms in experimental studies with the Milwaukee water supply found the aerogenes type less resistant particularly to chlorination, but that on subsequent storage these forms became the predominating colon type.

TABLE XLVI. RELATIVE INCIDENCE OF COLI AND AEROGENES SECTIONS IN RAW AND TREATED WATER SUPPLY OF MILWAUKEE.
(After Ellms 1920)

	Lake	Settled	Filtered		Chlorinated		
			Filter A	Filter B	Filter A	Filter B	Basin
No. of strains	710	655	288	294	28	43	13
%V.-P.+	44	40	40	33	29	26	70
%V.-P.—	56	60	60	65	71	74	30

Levine recorded the following experiences with the water supply of Dijon, France. The raw water examined daily for six months yielded 96 positive results for members of the colon group with *Bact. aerogenes* present in 16.6 percent of these. The tap samples during the same period (the water was treated with .07 to 0.1 part per million of chlorine, then stored in distribution reservoirs) showed only three positively confirmed tests, but *Bact. aerogenes* was present in each instance or 100 percent of the positively confirmed gas tubes.

Recently Hinman reported the relative incidence of these two groups in the water supply of Iowa City which is both filtered and chlorinated. In the raw water, out of 220 positively confirmed tests, aerogenes forms were present in 35 or 16 percent. In the treated water only 11 samples were confirmed for the colon group but of these 4 (36%) were *Bact. aerogenes.*

H. E. Jordan in a report on the Indianapolis water supply notes a seasonal variation in the relative resistance of these forms to chlorination which he found to be particularly effective against the coli section during the summer.

TABLE XLVII. THE VIABILITY OF CAPSULATED AND NON-CAPSULATED LACTOSE-FERMENTING ORGANISMS IN BOILED HARD WATER.
(After Wood 1919)

	Non capsulated	Capsulated Organisms	
Duration of Experiment	"Typical" *Bact. coli* V. P.—; M. R.+	Strain T. V.P.+;M.R.—;Indol— *Bact. aerogenes*	Strain N. D. V.P.+:M.R.—;Indol+ *Bact. aerogenes*
Start	1,250 per c. c.	580 per c. c.	Not enumerated, approximately the same as Strain T.
2 weeks	1 to 10 per c. c.	1 to 10 per c. c.	
4 weeks	Present in 100 c. c.	Present in 0.1 c. c.	
5 weeks	Absent from 100 c. c.	Present in 0.1 c. c.	
6 weeks	Absent from 100 c. c.	Present in 0.1 c. c.	
7 weeks		Present in 0.1 c. c.	
12 weeks		Present in 0.1 c. c.	Present in 1 c. c.
19 weeks		Present in 0.1 c. c.	
25 weeks		Present in 0.1 c. c.	
33 weeks		2,800 per c. c.	
39 weeks		2,000 per c. c.	
1 year		Present in 1 c. c.	Present in 1 c. c.
1 year, 4 months			Present in 1 c. c.
1 year, 9 months		Present in 1 c. c.	
2 years		Present in 1 c. c.	
3 years, 6 months			Still present

The evidence as to the effect of filtration and chlorination, particularly when followed by storage, on the relative prevalence of the coli and aerogenes types is meager and somewhat conflicting. Further observations on this phase are needed and would be of value. Clemesha states that the aerogenes group, for a while at least, multiplies in water. It would be in-

teresting and significant to know whether its increased prevalence in stored chlorinated water reported by Hinman, Ellms, H. E. Jordan, and the writer is due to such a secondary multiplication or merely to survival over the less viable *Bact. coli.*

That under certain conditions the *Bact. aerogenes* may multiply and survive for an incredibly long time in water is shown by the following experiment of Wood. He inoculated a hard water, which had been previously boiled and which contained no measurable trace of saline or organic ammonia, with *Bact. coli* and *Bact. aerogenes* and determined their incidence and relative viability. The results are indicated in Table XLVII.

The *Bact. coli* disappeared quite rapidly; an initial count of 1,250 per c. c. decreasing to less than 1 in 100 c. c. after five weeks' storage. With the aerogenes strains, however, there was an increase from 580 to 2,800 per c. c. after 33 weeks' storage and the organism was still present in one experiment after 3½ years.

Ellms in his studies on the Milwaukee supply detected colon forms in the chlorinated stored water (basin samples) on only five of the experimental runs but in three of these the colon index (see table XLVIII) was considerably higher than in the chlorinated filter effluents.

TABLE XLVIII. COLON INDEX IN CHLORINATED FILTERED AND STORED WATER.

(Calculated from report of Ellms 1920)

Operation period	Colon bacilli per liter			Ratio of basin to chlorinated filter effluents
	Filter A*	Filter B*	Basin	
9-8 —10-21, 1919	4	4	2	0.5
10-21—10-25, 1919	0	6	33	11.0
11-6 —11-12, 1919	74	61	50	.7
11-12—11-24, 1919	14	45	100	3.3
12-6 —12-9 , 1919	21	17	367	19.3
% cultures V.P.+	*29	26	70	

*Chlorinated filter effluents.

Taking into consideration that the filter effluents showed only 26-29 percent V. P. positive strains as compared with 70 percent after storage in the basin, the writer feels that the rise of the colon index in the latter was most likely due to multiplication of the aerogenes and cloacae forms.

That the colon index may increase on storage of a filtered chlorinated water may be shown quite strikingly from the report of the Indianapolis Water Company.

The incidence of colon bacilli in tap samples was at times 11 to 13 times as great as in the water works plant effluent.

TABLE XLIX. COLON GROUP PER 100 C. C. IN INDIANAPOLIS WATER SUPPLY
(Calculated from report of H. E. Jordon 1920)
Average for 3 years 1917-18-19.

	Plant effluent	Tap	Ratio of tap to plant effluent	* % Aerogenes in plant effluent
Jan.	2.1	3.1	1.5	30
Feb.	0.87	1.94	2.2	40
Mar.	0.72	0.83	1.2	66
Apr.	0.50	0.73	1.5	69
May	0.63	0.36	1.0	†87
June	0.37	1.33	3.6	58
July	0.21	2.87	13.5	75
Aug.	0.13	1.47	11.0	82
Sept.	0.33	4.40	13.2	50
Oct.	0.47	1.67	3.6	43
Nov.	0.47	0.93	2.0	45
Dec.	2.17	1.50	0.7	19

*Average for five years includes two years before chlorination.

†In two years before chlorination ratio of basin to plant effluent was 13.7.

In general the proportion of aerogenes (M. R. —-V. P. +) strains in the plant effluent was greatest in the warmer months. The relative incidence of aerogenes and coli types in the tap samples is not stated but it will be noticed from Table XLIX. that secondary multiplication was most marked in July, August and September. The temperature of the water at these times is quite likely to be very near the optimum for growth of *Bact. arogenes* and closely allied bacilli.

The foregoing observations are not sufficiently extensive to warrant final conclusions. They nevertheless do indicate that under some conditions there may be a secondary rise in the colon index of a filtered or chlorinated water on storage, and that this is probably due to the growth of the aerogenes section, for the increase is associated with those months when the aerogenes types were relatively more numerous and when the temperature of the water was near the optimum for growth of these forms.

Are all Varieties and Species of the Aerogenes and Coli Sections of the Same Sanitary Significance? Although the aerogenes group is rare in human feces, it is nevertheless present in small numbers, and similarly *Bact. coli*, although comparatively infrequent, is occasionally encountered in presumably non-polluted sources. It certainly would be a great aid if some test could be devised which would distinguish the *Bact. aerogenes* from human feces and those from soils and grains, or which

would distinguish the *Bact. coli* of the human intestine from those in the soil or of animal intestinal origin.

Rogers suggested that fermentation of adonite was a convenient differential index for the separation of the human from the grain strains of *Bact. aerogenes*. Of 46 human strains, all fermented adonite, whereas of 111 strains isolated from grains, only 12.6 percent were adonite fermenters.

This differentiation was incorporated in the Standard Methods of 1917, which divided the *Bact. aerogenes* forms into two subgroups, (1) the adonite fermenting type supposedly of human origin and (2) the adonite non-fermenting type regarded of non-fecal origin.

Other investigators, however, do not agree as to the value of adonite for this purpose. Monfort assigns to adonite about the same significance as to dulcite, saying that it is of rather dubious importance and of little significance as an index of pollution. Winslow and Cohen, in a study of water of known sanitary quality, found no evidence to support the contention that adonite fermentation by the *Bact. aerogenes* was indicative of fecal origin. In fact, their results were exactly the converse; 59 percent of the aerogenes strains from polluted water fermented adonite, whereas 90 percent from non-polluted sources were adonite fermenters which would indicate that the adonite fermenters (fecal varieties) were more prevalent in non-polluted water.

Chen and Rettger report 152 (34%) of their 447 aerogenes strains from soil to be adonite fermenters. It would appear, therefore, that adonite fermentation can not be considered, for the present at least, a reliable criterion for the identificaiton of *Bact. aerogenes* of fecal origin.

In 1905, MacConkey first subdivided the colon group into separate species, suggesting that information may thereby be obtained as to the relative value of specific members of the group as indicators of pollution. Clemesha advocates strongly such consideration and points out that the dulcite non-fermenters (MacConkey's groups 1 and 4) are resistant in water, whereas the dulcite fermenting forms (groups 2 and 3 of MacConkey) are sensitive, dying off rapidly. He contends that for tropical water a distinction must be made between the resistant dulcite non-fermerters and the non-resistant dulcite fermenting organisms. Whether these suggestions are as applicable to the temperate climate of our country is doubtful except possibly the hottest season of the year or in our tropical possessions. In this connection it should he pointed out that the observations of Houston do not agree at all with those of Clemesha as to the relative resistance of the dulcite fermenters and non-fermenters. The so-called sensitive dulcite fractors were less prevalent while the supposedly resistant forms were found to be more numerous in raw than in stored or filtered water.

Levine suggests that there is some correlation between the species of the coli section and their source, and that the determination of species may have some bearing upon interpretation of analyses.

From a study of 333 colon strains isolated from various sources, as indicated in Table L., he observes with reference to the coli section that *Bact. communior* was extremely abundant in the horse (79%) and sheep

(72.8%) but much less frequent in the cow (30%) and pig (29%); whereas in sewage and man it was relatively rare (7.7 and 8% respectively).

TABLE L. DISTRIBUTION OF ORGANISMS FROM DIFFERENT SOURCES AMONG THE VARIOUS SPECIES AND VARIETIES.

		B. cloacae	B. aerogenes	B. communior	B. neapolitanus	B. coscoroba	B. coli		B. acidi-lactici		Total
							communis	immobilis	Gruenthali	immobili	
Soil	No	88	54	26	0	0	2	0	7	0	177
	%	49.7	30.5	14.7			1.1		4.0		
Horse	No	0	0	15	0	0	4	0	0	0	19
	%			79.0			21.0				
Sheep	No	0	0	16	0	5	1	0	0	0	22
	%			72.8		22.7	4.5				
Cow	No	0	0	6	4	0	9	0	1	0	20
	%			30.0	20.0		45.0		5.0		
Pig	No	0	0	9	0	1	11	1	9	0	31
	%			29.0		3.2	35.6	3.2	29.0		
Sewage	No	1	8	3	3	2	1	12	2	7	39
	%	2.6	20.5	7.7	7.7	5.1	2.6	30.8	5.1	17.9	
Man	No	0	0	2	0	1	5	5	1	11	25
	%			8.0		4.0	20.0	20.0	4.0	44.0	
Total		89	62	77	7	9	33	18	20	18	333

The *Bact. neopolitanum* was present only in bovine feces and sewage, comprising 20 percent of the bovine and 7.7 percent of the sewage.

Bact. coscoroba occurred as follows: sheep 22.7 percent, pig 3.2 percent, sewage 5.1 percent and human 4.0 percent.

The relation of sucrose fermentation and the source is especially emphasized. The sucrose fermenting strains are relatively uncommon in human feces, whereas they constitute the predominating type in animal feces and in the soil. This low incidence in human feces is confirmatory of the observations of numerous other investigators.

In this connection, it may be well to recall that when Durham suggested the name *B. coli communior* for the sucrose fermenting variety because of its greater prevalence, his observations were based on the intestinal contents of animals for which this fact holds true, but, as has been pointed out above, the sucrose positive strains are relatively scarce in man.

Bact. coli, like *Bact. communior* was isolated from all of the sources tested, but a rather distinct correlation with the source is observed with the varieties *Bact. coli-communis* and *Bact. coli-immobilis.* The former comprise 1.1 percent of soil; 21 percent of horse; 4.5 percent of sheep; 45 percent of cow; 35.6 percent of pig; 2.6 percent of sewage; and 20 percent of human strains. *Bact. coli-immobilis* was not obtained from

the soil, horse, sheep or cow, but it made up 3.2 percent of the pig, 30.8 percent of the sewage, and 20 percent of the human strains.

TABLE LI. FERMENTATION OF SUCROSE BY BACT.-COLI-LIKE BACTERIA FROM HUMAN FECES.

Investigators	Number of organisms studied	Number of sucrose fermenters	Percentage of sucrose fermenters
Houston, 1902-3	100	30	30
MacConkey, 1905 and 1909	419	142	33.9
Ferreira, Horta, Paredes, 1908	117	44	37.6
Winslow and Walker 1907	25	8	32
Howe, 1912	540	324	60
Clemesha, 1912	1200	348	29
Browne, 1915	175	20	11.3
Levine, 1916	25	3	12
Total	**2601**	**919**	**35.3**

Bact. acidi-lactici was not obtained from the horse nor sheep, and only rarely from the cow (5.0%) or soil (4.0%). The motile variety *Bact. acidi-lactici var. Grunthali* was particularly abundant among the pig cultures (29%) and rare in sewage (5.1%) and man (4%). The non-motile *Bact. acidi-lactici var. immobili* was restricted to man and sewage entirely, comprising 44 percent of the human and 17.9 percent of the sewage strains.

If the sucrose negative forms are more indicative of human pollution it would be anticipated that they would be more prevalent in the more intensely polluted waters. Observations by the writer on 78 samples collected in France were as follows:

Among 34 samples in which the coli section was present in 1 c. c. or smaller quantities, sucrose negative strains were detected in 23 or (68%), whereas of 44 samples containing the coli section only in 10 c. c. or larger quantities but 13 (29%) showed sucrose negative coli strains. That is, the more polluted supplies apparently did contain a greater proportion of sucrose non-fermenters. More extensive work on the correlation of species of colon bacilli with the source, character of pollution, and history of waters is certainly desirable.

Resume. From considerations of the requirements for an index of pollution, the colon group appears to be a convenient and desirable one. It is not however, an ideal indicator for the species which it comprises are not all of equal sanitary significance.

The evidence seems to be clear and definite that the colon group comprises two subgroups or sections which are characteristically of different habitat; one, typified by *Bact. coli*, is present in large numbers in feces and sewage, whereas the other, exemplified by *Bact. aerogenes*, is rare in

such objectionable matter but predominates on the presumably harmless soil and grains. The aerogenes group, as indicated by laboratory experiments and observations in the field, is much more viable in water, where it will persist for long periods, and seems capable of growing, to some extent, in stored treated supplies.

It is not possible from our present limited knowledge of these two groups to put forth any definite rules for interpreting the significance of their presence in water, but it is felt that the presence of *Bact. aerogenes* alone should not be regarded as objectionable as is the presence of the *Bact. coli* in equal numbers. If the sanitary survey is favorable and there is no evidence of the true *Bact. coli* types in a water supply under different weather conditions, then a considerably greater number of *Bact. aerogenes* may be tolerated.

The presence of *Bact. aerogenes* alone (i. e. not associated with *Bact. coli*) in a supply may indicate merely remote pollution or soil contamination which is not as objectionable and certainly not as dangerous as sewage pollution.

Differentiation of the coli and aerogenes types in routine water analysis is obviously desirable as it may assist in the detection of the probable source and nature of the contamination.

VI. THE SPORE FORMING LACTOSE FERMENTERS AND THEIR SIGNIFICANCE IN WATER ANALYSIS.

Spore forming lactose fermenters are not infrequently encountered in water. They are for the most part anaerobes resembling the *Cl. welchii (B. aerogenes capsulatus* of Welch) or the *Cl. enteritidis sporogenes* group of Kline, but recently spore formers capable of growing on aerobic plates have been reported and isolated by Meyers, Ewing and Ellms and Hinman and Levine. They interfere seriously with the presumptive test for the colon group.

Sanitary Significance. Very little is known as to the source, distribution, or pathogenicity of the aerobic sporing types. Ellms reports their presence in feces while Ewing emphasizes that they were present in water only after heavy rains, so that they may represent soil forms. No reliable conclusion can be drawn at present as to their sanitary significance.

The anaerobes may be frequently encountered, often in large numbers, in the intestinal tract of man. Kline and Houston report 30 to 2,200 *Cl.* (*enteritidis*) *sporogenes* per c. c. in sewage, whereas in waters of good quality such forms are often absent even from large volumes. These observations have sometimes led to the contention that the presence of *Cl.* (*enteritidis*) *sporogenes* or closely allied bacteria in water is indicative of fecal pollution. The employment of these organisms as indices of dangerous pollution appears to the author unwarranted, undesirable and impractical for,

1. They are not characteristic of the human intestine.
2. There is a very little correlation between the incidence of Sporogenes and Welchii types in water and the sanitary survey.
3. They are extremely resistant.

The anaerobic lactose fermenters are verv widely distributed in nature. They are encountered in large numbers in manures from various animals, in decomposing organic materials, and in the soil. They cannot be considered distinctive or characteristic of the human intestinal tract.

In surface waters, the number of spores is remarkably constant, quite independent of the degree of sewage pollution as indicated by sanitary inspection.

Thus Cumming reports "Unlike *B. coli* which varies many thousand percent, from several hundred per c. c. to less than 1 in 10 c. c., according to the intensity of pollution, these spores were found often in the best river water in 10 c. c. and seldom showed an average much above 4 or 5 per c. c. . . . ."

"Their number furnishes no clue to the degree of pollution and purification as does the number of *B. coli.* . . . . ."

"The generally uniform distribution of organisms of this group in surface water, even in those not highly contaminated with sewage, and with no considerable increase in polluted waters, indicates that this group is not, as has sometimes been supposed, an organism characteristic of the intestine."

TABLE LII. CHARACTERISTICS OF LACTOSE FERMENTING ANAEROBES

Species	Motility	Gram Stain	Spores	Liquefaction of Gelatin	Liquefaction of Coagulated Serum	Milk	Meat Medium	Pathogenicity
Cl. welchii (Syn.; *B. aerogenes capsulatus*; *B. phlegmonis emphysematosae*; *B. perfringens*; *B. enteritidis sporogenes*; *Bacillus of Achalme*)	—	+ y — o	Large; oval with slightly flattened ends; central or subterminal. Spores formed only in sugar free media, rich in protein, such as coagulated serum, alkaline egg fluid, and casein broth.	+	Not liquefied; speres formed, filaments and involution types occur.	Stormy fermentation; very rapid clotting with evolution of gas; acid reaction; clot torn by gas.	Gas, pink color; sharp butyric acid odor; ne blackening.	*Pathogenic* for guinea pigs, pigeons and mice; less so fer rabbits. Many strains of low pathogenicity. Specific soluble toxin.
Cl. oedematis (Syn: *Vibrion septique* *B. oedematis maligni*)	+	+	Central or subterminal: formed readily in all media.	+	Net liquified; variations in morphology — citrens, navicular types, club forms, etc. present.	Acid and clot; seme gas may be formed; slow reaction, 3-6 days.	Gas; pink color which fades; no blackening.	*Pathogenic* for guinea pigs, pigeons, rabbits, and mice. Produces specific soluble toxin.
Cl. chauvoei (Syn: *B. chauvoei*)	+	+ y — o	Central or subterminal; readily formed.	+	Not liquified; navicular forms, etc., may be developed.	Acid; clot in 3-6 days; some gas may be evolved.	Gas; pink color which fades; ne blackening.	*Pathogenic* for mice and guinea pigs; rabbits relatively insusceptible. Produces specific agglutinins and soluble toxin.
Cl. aerofetidum (Syn: *B. aerofetidus*)	sl.	+ y — o	Spores oval and subterminal; not readily formed.	+	+	Clot and gas, 24-48 hrs. later some digestion	Putrid odor; red then blackening.	*Not pathogenic*
Cl. butyricum (Syn: *B. butyricus*)	+	+ y — o	Oval, usually central, but may also be subterminal.	—	—	Firm acid clot in 24 hrs.	Gas; no blackening.	*Not pathogenic*
Cl. multifermentans (Syn: *B.multifermentum*)	+	+	Central or subterminal.	—	—	Acid and clot.	Gas; no blackeuing.	*Not pathogenic*
Cl. tertium (Syn: *B. tertius*)	— or very faintly motile	+ y — o	Spores formed readily; they are oval and strictly terminal.	—	—	Acid and later clot.	Gas; no blackening.	*Not pathogenic*
Cl. sphenoides (Syn: *B. sphenoides*)	+	+ y — o	Spherical and terminal when fully developed.	—	—	Acid and sometimes a soft clot.	Little gas; no change in color.	*Not pathogenic*

y=young cultures o=old cultures

Furthermore, as these anaerobes are extremely resistant they would persist in water for considerable periods beyond any possible danger of transmission of intestinal disease (as typhoid, dysentery, cholera) the organisms of which (being non spore bearing) die off quite rapidly in water. Again they resist disinfection and other methods of water purification, which experience has shown efficient and sufficient for elimination of disease producing microorganisms. The presence of these spore formers in a treated water is entirely insignificant as an index of danger from typhoid or similar diseases.

It is conceivable, however, that some members of the Welchii group may themselves be responsible for intestinal disturbances. Herter in this country, and Klein in England have considered *Cl. welchii* as a cause of diarrhea in children. Such affections have been traced in a few instances to milk.

As an index of specific infection, the determination of the Welchii group in water analysis may possibly be of value, but if employed for this purpose, it would obviously be necessary to first determine the particular species or variety which is an intestinal pathogen. The terms *Cl. welchii* and *Cl.* (*enteritidis*) *sporogenes*, as employed by water works opeartors and analysts, designate not a specific organism but the whole group of anaerobic lactose fermenters. Some of these are known to be responsible for serious wound infections but many are harmless and no specific strain has been definitely proven to be pathogenic when restricted to the intestinal tract.

The Anaerobic Sporing Lactose Fermenting Species and Varieties. These are for the most part rather long rods, possessing central or subterminal spores usually slightly larger than the diameter of the cell. During the war considerable work on anaerobes was in progress and an extensive literature has developed which was carefully summarized in the Special Report No. 39 of the Medical Research Committee of England.

Table LIII., taken for the most part from the foregoing report summarizes the characteristics of the lactose fermenting anaerobes.

TABLE LIII. FERMENTATION REACTION OF LACTOSE FERMENTING SPORING ANAEROBES.

Species	Glu.	Lev.	Gal.	Mal.	Lac.	Suc.	Man.	Gly.	Dul.	Sal.	Starch	Inulin
*Cl. welchii**	+	+	+	+	+	+	—	±	—	—	+	±
Cl. oedematis	+	+	+	+	+	—	—	—	—	+		—
Cl. chauvoei	+	+	+	+	+	+	—	—	—	—		—
Cl. aerofetidum	+	+	+	+	+	—	—	—	—	+	—	—
Cl. butyricum	+			+	+	+	—	—	—	—	+	—
Cl. multi-fermentans	+			+	+	+	—	+	—	+		+
Cl. tertium	+	+	+	+	+	+	+	—	—	+	+	—
Cl. sphenoides	+	+	+	+	+	±	±	—	—	+	±	—

*Four types have been differentiated on inulin and glycerol fermentation.
Type I. Inulin+, glycerol+. Type II. Inulin—, glycerol+. Type III. Inulin+, glycerol —. Type IV. Inulin—, glycerol—.

KEY TO THE MORE COMMON SPORE PRODUCING LACTOSE FERMENTING ANAEROBES.

I. Gelatin liquefied (generally pathogenic).
- A. Coagulated serum, liquefied (non-pathogenic) — 1. *Cl. aerofetidum*
- B. Coagulated serum not liquefied (pathogenic)
  - 1. Non motile — 2. *Cl. welchii*
  - 2. Motile
    - a. Sucrose fermented salicin not attacked — 3. *Cl. chauvoei*
    - b. Sucrose not attacked, salicin fermented — 4. *Cl. oedematis*

II. Gelatin not liquefied (non pathogenic)
- A. Non motile or very faintly motile — 5. *Cl. tertium*
- B. Motile
  - 1. Salicin not fermented — 6. *Cl. butyricum*
  - 2. Salicin fermented
    - a. Glycerol and inulin fermented — 7. *Cl. multifermentans*
    - b. Glycerol and inulin not fermented. — 8. *Cl. sphenoides*

Creel made a very interesting and, from the standpoint of the presumptive test, an important observation in his study of drinking waters on railroad trains. He found two types of anaerobes which he designates "Group A and B" respectively. "Group A" comprises very long Gram-negative bacili (6-9 microns long and about 0.5 microns in width) whose spores are very much larger than the diameter of the cell. It is particularly significant to note that this group grows very rapidly producing considerable gas in lactose broth, under anaerobic conditions, but that no gas is evolved in the lactose bile medium. Creel's anaerobes of "Group B" were found to be non-motile, capsulated, resembling in general *Cl. welchii*. This group will not produce gas in lactose broth unless the medium has been freshly boiled or steamed in the Arnold until all air is expelled but in ordinary lactose bile, on the other hand, gas is formed very readily.

These observations may explain the controversy as to the relative value of lactose broth and bile as a presumptive test for the colon group. In dealing with waters containing anaerobes of "Group A" lactose bile will prove more reliable whereas if "Group B" is the predominating anaerobe then lactose broth will be found to give a higher proportion of confirmed presumptive tests.

Isolation of Anaerobes. Several methods have been suggested and employed for the isolation of anaerobic spore forming lactose fermenters from water. That originally described by Kline is as follows:

Milk is inoculated with the water under examination and heated for 10 minutes at 80° C. to destroy vegetative cells. The tube is then cooled, made anaerobic, and incubated at the body temperature. In about 24 to 36 hours, a characteristic, so-called "sporogenes" reaction will be ob-

served. This is described by Kline as follows:

"The cream is torn or altogether dissociated by the development of gas so that the surface of the medium is covered with stringy, pinkish-white masses of coagulated casein enclosing a number of gas bubbles. The main portion of the tube formerly occupied by the milk now contains a colorless, thin, watery whey, with a few casein lumps adhering here and there to the sides of the tube. When the tube is opened, the whey has a smell of butyric acid and is acid in reaction. Under the microscope the whey is found to contain numerous rods, some motile, others motionless."

The method employed by Creel, which was said to be very efficient for isolation in pure culture is given herewith.

Petri dishes are selected having covers considerably larger than the inner plates. Agar is poured into the inner dish, allowed to harden and then inoculated by smearing over the surface with material from a broth or lactose bile tube suspected of containing an anaerobe. The inoculated plate is then inverted into the large cover. Three grams of pyrogallic acid are placed in the cover, one c. c. saturated potassium hydroxide is inserted with a pipette, and the two dishes are immediately sealed with melted paraffin. Incubation is at the body temperature.

In the Standard Methods of Water Analysis A. P. H. A. 1912, a detailed procedure for the detection of these anaerobes is recommended as given below:

"*B. sporogenes** is indicated by a vile odor which is produced in the liver broth fermentation tubes used in the regular test for general gas forming bacteria. The specific tests are made as follows:

1. Inoculate various dilutions (usually 0.1, 1.0 and 10.0 c. c.) of water, or of sewage in higher dilutions, into fermentation tubes containing liver broth and incubate for 24 hours at 37° C. If *B. sporogenes* is present in the dilutions used, there will be vigorous gas formation, accompanied by an offensive odor, and numerous large spores will be present.

2. Transfer the entire contents of each tube showing gas plus characteristic odor into separate sterile Erlenmeyer flasks or large test tubes and heat to 80° C. for 10 minutes.

3. One (1) c. c. (not more) of broth containing sediment is withdrawn from the bottom of each of the flasks or tubes which have been heated, and is planted separately into a second set of sterile liver broth fermentation tubes and incubated for 24 hours at 37° C. after which time gas formation and characteristic odor will again be observed. Microscopic examination will reveal the presence of numerous large sluggishly motile bacilli containing spores. Usually *B. sporogenes* is now present in pure culture.

4. A stab culture made from this 24 hour liver broth culture into dextrose liver gelatine or nutrient gelatin will demonstrate the presence of *B. sporogenes* by the following characteristic growth. After 48 hours incubation at 20° C., a distinct anaerobic growth will be observed begin-

*Term employed to designate the Welchii group and not a specific organism.

ning about two centimeters below the surface. Liquefaction will be well advanced and gas bubbles will accumulate at the top of the liqufied area.

5. In order to obtain colonies of *B. sporogenes* on agar plates it is necessary to transplant a few drops of broth and sediment from the second set of fermentation tubes, in step 3, into a third set of tubes and incubate for three to five hours at 37° C. After that period a distinct anaerobic growth will be observed in the closed arm, and a few bubbles of gas will be seen at the top. The *B. sporogenes* is now in the vegetative state and this is the only condition in which it will grow on the plates.

The contents of the closed arm are transferred to the open bulb by tilting forward, and plated in dilutions of 1.0 to .00001 c. c. on dextrose liver agar, and incubated for 12 to 18 hours in hydrogen at 37° C. Typical colonies will then be visible consisting of one or more gas bubbles surrounded by a delicate white fringe. The plate cultures also have a disagreeable cheesy odor.

6. From one of these typical colonies a deep stab culture is made into dextrose liver agar and incubated for 24 hours. A distinct anaerobic growth will be observed along the line of puncture and sometimes the agar is split into two or three layers by the gas evolved.

7. A sub-culture may also be made into litmus milk and incubated for 48 hours, anaerobically, after which time there will be a complete separation of curd and whey and a strong odor of butyric acid. Sometimes the curd adheres to the sides of the tubes and has a peculiar shredded appearance."

It should be emphasized that none of the foregoing methods will yield all the sporing lactose fractors. Detailed procedures for isolation of specific forms together with the special media necessary are described in the English Report on Anaerobic Bacteria and Infections, to which reference has already been made.

The Aerobic Sporing Lactose Fermenters. Meyers, in 1918, isolated a spore forming lactose fermenter capable of growing on the surface of Endo agar, from the water supplies of Newport and Covington, Kentucky and from tannery wastes.

The organism is Gram negative, grows readily on Endo agar, producing a red colony in 24 hours, which shows a distinct metallic luster after 48 hours, and in Clark and Lubs medium it is alkaline to Methyl Red and positive for the Voges Proskauer Reaction. The more important characteristics as detailed by Meyers are:

Agar slant. Growth quite distinctive. At 37° C., in 24 hours, thin transparent veil-like growth over entire surface except the very top. Growth lobate along upper edge. Microscopically—in 24 hours mainly vegetative forms, in 48 hours spore-bearing forms and later only free spores.

Endo's plates at 37° C. In 24 hours, colonies pink with red center, irregular contour, one to two m. m. diameter, little or no sheen. Colonies 48 hours, deep red, much sheen in colonies and surrounding medium. Latter point distinctive.

Gelatin stab at 20° C. In 48 hours, beginning liquefaction; in 72 hours liquefaction infundibuliform, slight precipitate.

Carbohydrates. In standard extract broth to which has been added one percent of the following carbohydrates, acid and gas are formed: (1) glucose, (2) laevulose, (3) raffinose, (4) maltose, (5) sucrose, (6) lactose, (7) inulin, (8) starch, (9) glycerol, (10) mannitol. No acid or gas and little growth in dulcite broth, which remains clear and limpid. In other broths gas usually appear in 24 hours. Media uniformly clouded, slight stringy precipitate, no pellicle. Media 48 hours, slightly viscous.

Clark and Lubs. Typical reaction of 'Grain' type coli, in 48 hours at 37° C., i. e. reaction alkaline to methyl red, Voges-Proskauer test positive.

Indol production in 1 percent peptone, four days at 37° C. No indol detected when tested for by the nitrite and by Ehrlich's para-dimethyl-amido-benzaldehyde method.

Glucose-neutral-red broth. Same reaction as *Bact. coli* i. e. yellow fluorescence with gas formation.

Litmus milk at 37° C. In 24 hours acid, in 48 hours partially reduced, coagulated with extrusion of whey; beginning digestion of curd.

Lactose bile at 37° C. In 96 hours, no gas or growth.

Chromogenesis. None.

Ewing, in 1919, reported a similar bacillus in the water supply of Baltimore and noted that its presence was associated with heavy rainfall.

TABLE LIV. ELIMINATON OF SPURIOUS PRESUMPTIVE TESTS.
(After Hall and Ellefson 1918)

Series	Gentian violet concentration	Positive presumptive tests	Colon group present	% Presumptive tests confirmed
A	None	21	12	57.1
	1-100,000	20	15	75.0
B	None	44	26	59.1
	1-20,000	33	27	81.8
C	None	85	58	68.2
	1-20,000	74	61	82.4
D Samples heated 60°.C 30 min.	None	81	3	3.7
	1-20,000	5	1	20.0

Inhibition of Growth of Spore Formers. Hall has suggested the use of gentian violet in the lactose broth presumptive test tube to eliminate spurious presumptive tests. In a series of examinations with water and pure cultures, he found that 1-20,000 to 1-100,000 gentian violet in lactose broth exerted but little inhibitory influence on the growth of *Bact. coli*; whereas the anaerobes were almost completely checked.

Table LIV. shows the marked inhibitory action of gentian violet on the anaerobes. Not only are spurious presumptive tests eliminated but the total of successful isolations of colon bacilli is increased. This

would indicate that the presence and growth of the Welchii group is detrimental to the successful isolation of *Bact. coli.* This work of Hall and Ellefson has recently been confirmed by Wagner and Monfort.

Ellms (1919) reports sporing lactose decomposing aerobes from the water supply of Milwaukee, and the feces of children. These strains differ from those of Meyers and Ewing in that they are Gram positive and are acid to methyl red. The importance of these aerobic spore formers to the bacteriologist and engineer is apparent. Being much more resistant than the non-sporing colon group, they would naturally survive the ordinary methods of water treatment, and as they are capable of growing aerobically, they may be mistaken for *Bact. coli, Bact. aerogenes,* etc., in the ordinary routine examination. Their presence in a water may conceivably account for the apparently poor results sometimes obtained in purification processes.

Muer and Harris, of the Mount Prospect Laboratory, observed that in lactose peptone bile a concentration of 1-700 to 1-1000 brilliant green would not appreciably affect the volume of gas produced by *Bact. coli* in seven days, whereas *Cl. welchii* would not produce gas until the brilliant green was diluted to 1-50,000. This observation is quite remarkable for, as is well known, brilliant green is frequently used to inhibit *Bact. coli.* The writer has observed a dilution of even 1-2,000,000 has a marked inhibitory effect on the rate of growth of *Bact. coli* in peptone water. Their results, however, are very distinct and significant. Probably the bile reacts in some way with brilliant green. It is well known, for example, that in eosin brilliant green agar a much higher concentration of brilliant green can be employed without affecting *Bact. typhi* than in the Andrade brilliant green medium of Krumwiede. The following table shows very clearly the inhibitory action of brilliant green on the anaerobic spore formers. It appears also that the growth of the anaerobes interferes with the isolation of *Bact. coli.* In 17 samples *Bact. coli* was isolated when brilliant green lactose bile was employed but was missed when plain lactose peptone bile was used.

TABLE LV. COMPARISON OF 115 SAMPLES OF WATER PLANTED IN BOTH PLAIN LACTOSE PEPTONE BILE AND BRILLIANT-GREEN LACTOSE BILE.
(After Muer and Harris 1920)

Medium	Number of dilutions from which *Bact. coli* was isolated	Number of dilutions from which *Cl. welchii* was isolated
Plain lactose-peptone bile	34	18
Brilliant-green lactose bile	51	0

The chief interest of the water bacteriologist in these spore formers is that the anaerobes interfere and confuse the presumptive test rendering confirmation necessary, while the aerobic forms complicate the confirmatory test as well, making it essential to resort to more detailed identification tests where there is reason to suspect this type present.

APPENDIX A. ROUTINE METHODS OF WATER ANALYSIS AND THE COLON INDEX.

Although the American Public Health Association has been issuing standard methods of water analysis for some fifteen years, there is still a marked lack of uniformity in the methods employed in different laboratories. Thus Norton (1918), in a tabulation of 23 laboratories, found that 13 employed lactose broth, 8 lactose bile, 1 lactose and dextrose broth. and 1 lactose agar and dextrose broth for primary inoculation or preliminary enrichment. The most commonly employed routine methods are given here.

I. The Treasury Department Standard for the Examination of Water on Interstate Common Carries. The following method for the examination of water on Interstate common carriers has been formulated by a committee of prominent sanitarians. The permissible limits of bacteriological impurity are stated as follows:

1. The total number of bacteria developing on standard agar plates, incubated 24 hours at 37° C., shall not exceed 100 per cubic centimeter; provided, that the estimate shall be made from not less than two plates, showing such numbers and distribution of colonies as to indicate that the estimate is reliable and accurate.

2. Not more than one out of five 10 c. c. portions of any sample examined shall show the presence of organisms of the *Bacillus coli* group when tested as follows:

(a) Five 10 c. c. portions of each sample tested shall be planted, each in a fermentation tube containing not less than 30 c. c. of lactose peptone broth. These shall be incubated 48 hours at 37° C. and observed to note gas formation.

(b) From each tube showing gas, occupying more than five percent of the closed arm of the fermentation tube, plates shall be made after 48 hours' incubation, upon lactose litmus agar or Endo's medium.

(c) When plate colonies resembling *B. coli* develop upon either of these plate media within 24 hours, a well-isolated characteristic colony shall be fished and transplanted into a lactose-broth fermentation tube, which shall be incubated at 37° C. for 48 hours.

For the purpose of enforcing any regulations which may be based upon these recommendations the following may be considered sufficient evidence of the presence of organisms of the *Bacillus coli group.*

Formation of gas in fermentation tube containing original sample of water (a).

Development of acid-forming colonies on lactose-litmus-agar plates or bright red colonies on Endo's medium plates, when plates are prepared as directed above under (b).

The formation of gas, occupying 10 percent or more of closed arm of fermentation tube, in lactose peptone broth fermentation tube inoculated with colony fished from 24 hour lactose litmus agar or Endo's medium plate.

These steps are selected with reference to demonstrating the presence in the samples examined of aerobic lactose-fermenting organisms.

3. It is recommended, as a routine procedure, that in addition to five 10 c. c. portions, one 1 c. c. portion, and one 0.1 c. c. portion of each sample examined be planted in a lactose peptone broth fermentation tube, in order to demonstrate more fully the extent of pollution in grossly polluted samples.

4. It is recommended that in the above-designated tests the culture media and methods used shall be in accordance with the specifications of the committee on Standard Methods of Water Analysis of the American Public Health Association, as set forth in "Standard Methods of Water Analysis" (A. P. H. A., 1912).

II. English Procedure (After Savage). Add 0.1 and 1.0 c. c. of water respectively to tubes of lactose bile salt broth in double tubes. Add 10 c. c. to a similar tube, but containing lactose bile salt broth of double strength. To the remainder in the bottle, after all the different amounts of water have been withdrawn for the different parts of the examination, add the contents (about 10 c. c.) of a tube of four times strength neutral red broth. Replace the glass stopper. Four times strength bile salt broth may be used, and, if the examination is for *B. coli* alone, is preferable, but by using neutral red broth the mixture is also available for the examination for streptococci.

If a 2-ounce sample is collected, the amount remaining in the bottle will be about 30 c. c. If a large sample of water is collected, then 50 c. c. should be added by sterile pipette to a tube of four times strength neutral red broth large enough to hold the added water.

The tubes are labeled, incubated at 37° C., and examined after 24 and after 48 hours.

If the 0.1, 1.0, and 10 c. c. tubes show no gas after 48 hours, it can be assumed that *B. coli* is absent in these amounts. Then, in every case, the larger amount (i. e. the 30 c. c. in the bottle) should be examined for this organism. The alteration of the red color to yellow, with the presence of fluorescence, is an indication of the probable presence of *B. coli.*

If gas is present in the tubes containing smaller amounts, use the one showing gas in the tube with the least quantity of added water for inoculating plates of solid media. In this way it can be definitely ascertained whether *B. coli* is present or absent in 50 c. c. or less, and if present, approximately in what numbers.

To isolate the *B. coli* group organism, a trace of the positive tube selected is distributed over the surface of a plate containing neutral red lactose bile salt agar (L. B. A.), fuchsin agar or some other medium selected. L. B. A. is recommended as most suitable. Several colonies should be subcultivated and worked out.

Subcultivation upon or in the following five media is recommended for routine work, i. e.:

(a) Gelatine slope (for morphology, motility, cultural appearance, and liquefaction).

(b) Litmus-milk at 37° C.
(c) Lactose-peptone litmus solution (in a double tube).
(d) Peptone water (for indol production).
(e) Saccharose peptone litmus solution (in a double tube).

III. American Public Health Association. Standard Method. The 1920 report of the Committee on Standard Methods of Water Analysis of the American Public Health Association suggested the following:

It is recommended that the *B. coli* (colon)* group be considered as including all non-spore-forming bacilli which ferment lactose with gas formation and grow aerobically on standard solid media.

The formation of 10 percent or more gas in a standard lactose broth fermentation tube within 24 hours at 37° C. is presumptive evidence of the presence of members of the *B. coli* group, since the majority of the bacteria which give such a reaction belong to this group.

The appearance of aerobic lactose-splitting colonies on lactose-litmus-agar or Endo's medium plates made from a lactose-broth fermentation tube in which gas has formed confirms to a considerable extent the presumption that gas-formation in the fermentation tube was due to the presence of members of the *B. coli* group.

To complete the demonstration of the presence of *B. coli* as above defined, it is necessary to show that one or more of these aerobic plate colonies consists of non-spore-forming bacilli which, when inoculated into a lactose-broth fermentation tube, form gas.

It is recommended that the standard tests for the *B. coli* group be either (a) the Presumptive, (b) the Partially Confirmed, or (c) the Completed test as hereafter defined, each test being applicable under the circumstances specified.

A. Presumptive Test. 1. Inoculate a series of fermentation tubes with appropriate graduated quantities of the water to be tested. In every fermentation tube there must always be at least three times as much medium as the amount of water to be tested. When necessary to examine larger amounts than 10 c. c. as many tubes as necessary shall be inoculated with 10 c. c. each.

2. Incubate these tubes at 37° C. for 48 hours. Examine each tube at 24 and 48 hours, and record gas-formation. The records should be such as to distinguish between:

(a) Absence of gas-formation.

(b) Formation of gas occupying less than 10 percent of the closed arm.

(c) Formation of gas occupying more than 10 percent (10%) of the closed arm.

More detailed records of the amount of gas formed, though desirable for purposes of study, are not necessary for carrying out the standard tests prescribed.

*Parenthesis author's.

3. The formation within 24 hours of gas occupying more than 10 per cent. (10%) of the closed arm of fermentation tube constitutes a **positive presumptive test.**

4. If no gas is formed in 24 hours, or if the gas formed is less than 10 percent (10%), the incubation shall be continued to 48 hours. The presence of gas in any amount in such a tube at 48 hours constitutes **a doubtful test,** which in all cases requires confirmation.

5. The absence of gas formation after 48 hours' incubation constitutes a **negative test.** (An arbitrary limit of 48 hours' observation doubtless excludes from consideration occasional members of the *B. coli* group which form gas very slowly, but for the purposes of a standard test the exclusion of these occasional slow gas forming organisms is considered immaterial).

B. Partially Confirmed Test. 1. Make one or more Endo's medium or lactose-litmus-agar plates from the tube which, after 48 hours' incubation, shows gas formation from the smallest amount of water tested. (For example, if the water has been tested in amounts of 10 c. c., 1 c. c., and 0.1 c. c. gas is formed in 10 c. c., and 1 c. c., not in 0.1 c. c. the test need be confirmed only in the 1 c. c. amount).

2. Incubate the plates at 37° C., 18 to 24 hours.

3. If typical colon-like red colonies have developed upon the plate within this period, the confirmed test may be considered positive.

4. If, however, no typical colonies have developed within 24 hours, the test cannot yet be considered definitely negative, since it not infrequently happens that members of the *B. coli* group fail to form typical colonies on Endo's medium or lactose-litmus-agar plates, or that the colonies develop slowly. In such case, it is always necessary to complete the test as directed under "C" 2 and 3.

C. Completed Test. 1. From the Endo's medium or lactose-litmus-agar plate made as prescribed under "B", fish at least two typical colonies, transferring each to an agar slant and a lactose broth fermentation tube.

2. If no typical colonies appear upon the plate within 24 hours, the plate should be reincubated another 24 hours, after which at least two of the colonies considered to be most likely *B. coli*, whether typical or not, shall be transferred to agar slants and lactose broth fermentation tubes.

3. The lactose broth fermentation tubes thus inoculated shall be incubated until gas formation is noted; the incubation not to exceed 48 hours. The agar slants shall be incubated at 37° C. for 48 hours, when a microscopic examination shall be made of at least one culture, selecting one which corresponds to one of the lactose broth fermentation tubes which has shown gas-formation.

The formation of gas in lactose broth and the demonstration of non-spore-forming bacilli in the agar culture shall be considered a satisfactory completed test, demonstrating the presence of a member of the *B. coli* group.

The absence of gas-formation in lactose broth or failure to demonstrate non-spore-forming bacilli in a gas-forming culture constitutes a negative test.

APPLICATION OF PRESUMPTIVE, PARTIALLY CONFIRMED, AND COMPLETED TESTS.

A. The Presumptive Test. When definitely positive, that is showing more than 10 percent (10%) of gas in 24 hours, is sufficient:

(a) As applied to all except the smallest gas-forming portion of each sample in all examinations.

(b) As applied to the smallest gas-forming portion in the examination of sewage or of water showing relatively high pollution, such that its fitness for use as drinking water does not come into consideration. This applies to the routine examinations of raw water in connection with control of the operation of purification plants.

2. When definitely negative, that is showing no gas in 48 hours, is final and therefore sufficient in all cases.

3. When doubtful, that is showing gas less than 10 percent (10%) (or none) in 24 hours, with gas either more or less than 10 percent in 48 hours, must always be confirmed.

B. The Partially Confirmed Test. 1. When definitely positive, that is, showing typical plate colonies within 24 hours, is sufficient:

(a) When applied to confirm a doubtful presumptive test in cases where the latter, if definitely positive, would have been sufficient.

(b) In the routine examination of water-supplies where a sufficient number of prior examinations have established a satisfactory index of the accuracy and significance of this test in terms of the completed test.

2. When doubtful, that is, showing colonies of doubtful or negative appearance in 24 hours, must always be completed.

C. The Completed Test. The completed test is required as applied to the smallest gas-forming portion of each sample in all cases other than those noted as exceptions under the "presumptive" and the "partially confirmed" tests.

The completed test is required in **all** cases where the result of the confirmed test has been doubtful.

IV. Modification of A. P. H. A. Method. The following procedure, which is a modification of the A. P. H. A. method has proved very satisfactory and convenient in the hands of the author.

PROCEDURE FOR THE EXAMINATION OF WATER. (BACTERIOLOGICAL)

	Steps in procedure	Further tests required
A	1. Plate *two* 1 c. c. and *one* 0.1 c. c. or other appropriate portions of the sample on plain agar, and incubate for 24 hours at 37° C. 2. Inoculate 10 c. c., 1 c. c. or other portions of the sample into lactose broth (or lactose peptone water). Incubate at 37° C. 3. **Optional if a very rapid result is necessary.** 8 to 10 hours after incubation perform a **Preliminary Confirmatory test** as follows: Divide an Eosine-methylene blue, (or Endo, or Litmus-lactose) agar plate into sectors. Streak out a drop or loop of a fermentation tube containing 10 c. c. of the sample on one of the sectors, and in a similar manner streak out other fermentation tubes on the remaining sectors. Incubate at 37° C.	
B	1. Count agar plates made the previous day, record and discard petri dishes. 2. Record presence or absence of gas after 24 hours incubation as follows: 10% or more,—**Positive**; less than 10%,—**Doubtful**; no gas,—**Negative.** 3. Gas formation (any amount) accompanied by a positive preliminary confirmatory test, constitutes what is known as a **Partially Confirmed** test for the colon group. 4. If 10% or more gas is formed, and the preliminary confirmatory test is negative or was not made, the result is regarded as a **positive presumptive** test for the colon group. 5. If less than 10% gas is formed, and the preliminary confirmatory test is negative or was not made, the result is regarded as a **doubtful presumptive test,** and **should** be confirmed.	None 'D' except for sewage or raw water 'D'
C	1. If no gas was formed after 24 hours' incubation, but is present after 48 hours, the test is regarded as doubtful and inconclusive. **This must** be confirmed.	'D'

PROCEDURE FOR THE EXAMINATION OF WATER. (BACTERIOLOGICAL)

	Steps in procedure	Further tests required
D	1. To confirm the presence of the colon group in a tube showing gas, streak out a loop of the medium onto eosine methylene blue agar (or Endo or litmus-lactose agar.) Incubate overnight at 37° C.	
	(a) Presence of characteristic colonies on the agar constitutes a **Partially Confirmed Test** for the colon group. Record whether the colony resembles the coli or aerogenes sections.	None
	(b) If no growth develops on the agar plate, it is considered that the gas produced in the fermentation tube was due to anaerobic organisms and not to coli-like forms. Record probably anaerobe.	None
	(c) If characteristic colonies of *Bact. coli* or its close allies are not present, the plates must be examined further before reporting absence of the colon group.	'E'
E	1. To determine whether *Bact. coli* or its close allies is present on a negative or questionable eosine methylene blue (Endo, or litmus-lactose) agar confirmatory plate.	
	(a) Pick one or two colonies which most resemble *Bact. coli* or *Bact. aerogenes* and plant into lactose broth (or lactose peptone water), and incubate for 24 hours at 37° C. If 10% or more gas is formed, record colon group present.	
	(b) A Gram stain should be made of the colony before inoculation into lactose broth to insure that a Gram-negative coli-like form in pure culture is being fished.	
	Clark and Lubs medium or glucose broth may be inoculated, if desired to test for the M. R. or V. P. reaction, respectively.	

THE COLON INDEX

Estimation of the Incidence of the Colon Group. It is apparent that all agree in the use of the preliminary enrichment method for the determination of the presence of the colon group but a moment's thought

will show that it is extremely difficult to estimate the number of colon forms present from the result obtained. Perhaps the point can best be illustrated by considering a specific example.

Suppose a sample of water was examined and gave the following result for colon types: 1 c. c.+; 0.1 c. c.+; and 0.01 c. c.—. What was the incidence of the colon group per unit volume? Should 10 colon bacilli present per c. c. be recorded? It might be said, as is commonly stated, that there were more than 10 but less than 100 colon forms, and yet it is conceivable that there might be less than 10. Assume that there were five *Bact. coli* per c. c. In that case, in taking out a 0.1 c. c. sample the analyst would be just as likely to catch a *Bact. coli* as to miss one. The mere detection of the organism in a sample is not necessarily a safe criterion for regarding the organism constantly present in that quantity of water. On the other hand, the absence of colon forms in 0.01 c. c. is no justification for stating that such organisms would be absent if another 0.01 c. c. sample were taken, for if there were 50 colon bacilli present per c. c., the analyst would be just as likely to catch an organism as to miss one in a single 0.01 c. c. sample. It is apparent, therefore, that from the analysis presented it is extremely difficult to express by a single figure, the number of colon types present per unit volume. If, however, instead of having taken one portion of each dilution ten had been employed, a very much closer approximation to the actual number of organisms could be made. Similarly if the dilutions indicated were taken on ten different days a reasonably close estimate could then be made of the average number of colon bacilli present during that period.

In water works operation, and for comparison of the efficiencies of different plants, we are not concerned with a single analysis but with a series of analyses extending over a long period, perhaps a month or a year. A number of methods have been suggested for calculating the incidence of the colon group or what is known as the **"Colon Index."**

The most commonly employed method, and the one recommended by the A. P. H. A. is that of Phelps. More complicated but probably more accurate methods are described by McCrady, Wolman and Weaver, and Stein.

The Phelps method is based on the assumption that the most probable number of organisms present in any specimen is the reciprocal of the highest positive dilution; thus in the above example (1 c. c.+; 0.1 c. c.+; 0.01 c. c.—) 10 colon forms per cubic centimeter would be considered the most probable number. To obtain the colon index for a month or a year it is merely necessary to add the reciprocals of the highest positive dilutions for the individual (daily or otherwise) tests and divide by the total number of tests; this will give the average, but not necessarily the most probable, number for the period under consideration. An example follows:

Day	Results of Daily Test				Probable incidence of colon group
	1 c. c.	0.1 c. c.	0.01 c. c.	0.001 c. c.	
1	+	+	—	—	10
2	+	+	+	—	100
3	+	+	—	—	10
4	+	+	—	—	10
5	+	+	—	—	10
6	+	—	—	—	1
7	+	+	—	—	10

Total (for estimating averages)....................151
Average of 7 tests....................21.6 per c. c.

In 1915 McCrady proposed a method for the calculation of the most probable number of colon bacilli from a series of fermentation results but his methods have not been employed because of the cumbersome calculations involved. Wolman and Weaver, in 1917, simplified the McCrady formula and presented some graphs by means of which the tedious computations are almost entirely removed.

If A=total number of tubes inoculated with 10 c. c.
B=total number of tubes inoculated with 1 c. c.
C=total number of tubes inoculated with 0.1 c. c.
a=number of 10 c. c. tubes positive
b=number of 1 c. c. tubes positive
c=number of 0.1 c. c. tubes positive
then x—the most probable number of colon bacilli per 100 c. c.—may be obtained by trial substitutions of values for x in the following equation.

$$100A - 10B - 1C = \frac{100a}{1-.9^x} - \frac{10b}{1-.99^x} - \frac{1c}{1-.999^x}$$

From the charts accompanying Wolman and Weaver's paper, the values for x corresponding to any proportion of positive tests may be read off directly when dealing with a **single dilution.** When concerned with several dilutions the above formula must be employed, but the values of $(1-.9^x)$, $(1-.99^x)$, and $(1-.999^x)$ for any assumed value of x are also given on these charts so that the actual mathematics involved is reduced to merely simple arithmetic.

Stein in 1918, from a consideration of the laws of probability, evolved a curve from which the colon index, together with its reliability could be read directly if (1) the number of observations (2) the proportion of positive tests, and (3) the size of the test samples, were known.

For further details the reader is referred to the original papers by Stein, M. F. Journal of Bacteriology, IV, 1919, p. 243, and Wolman and Weaver, Journal of Infectious Diseases, XXI., 1917, p. 287. In the Public Health Journal (Canadian) IX., 1918, p. 201, McCrady presents a set of tables for the interpretation of fermentation-tube results, which almost completely relieve the analyst of mathematical calculations.

APPENDIX B.—CULTURE MEDIA

Numerous mediums have been utilized in the bacteriological examination of water. The preparation of the more important is described here.

I. ADJUSTMENT OF REACTION OF CULTURE MEDIA (A. P. H. A. 1920)

1. Phenol Red Method for adjustment to a hydrogen-ion concentration of PH+ = 6.8-8.4. Withdraw 5 c. c. of the medium, dilute with 5 c. c. of distilled water, and add 5 drops of a solution of phenol red (phenol sulphone phthalein). This solution is made by dissolving 0.04 grams of phenol red in 30 c. c. of alcohol and diluting to 100 c. c. with distilled water.

Titrate with a 1:10 dilution of standard solution of NaOH (which need not be of known normality) until the phenol red shows a slight but distinct pink color. Calculate the amount of the standard NaOH solution which must be added to the medium to reach this reaction. After the addition check the reaction by adding 5 drops of phenol red to 5 c. c. of the medium and 5 c. c. of water.

2. Titration with phenolphthalein. (For the convenience of those who wish to retain the use of this method for the present it is given here, but it is recommended that as soon as possible the more accurate method of determining the hydrogen-ion concentration be substituted.)

In a white porcelain dish put 5 c. c. of the medium to be tested, add 45 c. c. of distilled water. Boil briskly for one minute. Add 1 c. c. of phenolphthalein solution (5 grams of commercial salt to one liter of 50 percent alcohol). Titrate immediately with a n/20 solution of sodium hydrate. A faint but distinct pink color marks the true end point. This color may be precisely described as a combination of 25 percent of red (wave length approximately 658) with 75 percent of white as shown by the disks of the standard color top made by the Milton Bradley Educational Co., Springfield, Mass.

All reactions shall be expressed with reference to the phenolphthalein neutral point and shall be stated in percentages of normal acid or alkali solutions required to neutralize them. Alkaline media shall be recorded with a minus (−) sign before the percentage of normal acid needed for their neutralization and acid media with a plus (+) sign before the percentage of normal alkali solution needed for their neutralization.

The standard reaction for culture media for water analysis shall be +1.0 percent, as determined by tests of the sterilized medium. As ordinarily prepared, broth and agar will be found to have a reaction between +0.5 and +1.0. For such media no adjustment shall be made. The reaction of media containing sugar shall be neutral to phenolphthalein. Whenever reactions other than the standard are used, it shall be so stated.

II. STANDARD STOCK MEDIA

A. Nutrient Agar (A. P. H. A., 1920). 1. Add 3 grams of beef extract, 5 grams of peptone and 12 grams of agar, dried for one-half hour

at 105° C. before weighing, to 1,000 c. c. of distilled water. Boil over a water bath until all agar is dissolved, and then make up the loss by evaporation.

2. Cool to 45° C. in a cold water bath, then warm to 65° C. in the same bath, without stirring.

3. Make up lost weight and adjust the reaction to a faint pink with phenol red, or if the phenolphthalein titration is used, and the reaction is not already between +0.5 and +1, adjust to +1.

4. Filter through cloth and cotton until clear.

5. Distribute in test-tubes, 10 c. c. to each tube, or in larger containers, as desired.

6. Sterilize in the autoclave at 15 pounds (120° C.) for 15 minutes after the pressure reaches 15 pounds.

B. Nutrient Gelatin (A. P. H. A., 1920). 1. Add 3 grams of beef extract and 5 grams of peptone to 1,000 c. c. of distilled water and add 100 grams of gelatin dried for one-half hour at 105° C. before weighing.

2. Heat slowly on a steam bath to 65° C until all gelatin is dissolved.*

3. Make up lost weight and adjust the reaction to a faint pink with phenol red, or if the phenolphthalein titration is used, and the reaction is not already between +0.5 and +1, adjust to +1.

4. Filter through cloth and cotton until clear.

5. Distribute in test-tubes, 10 c. c. to each tube, or in large containers as desired.

6. Sterilize in the autoclave at 15 pounds (120° C.) for 15 minutes after the pressure reaches 15 pounds.

C. Nutrient Broth. (A. P. H. A., 1920). 1. Add 3 grams of beef extract and 5 grams of peptone to 1,000 c. c. of distilled water.

2. Heat slowly on steam bath to at least 65° C.

3. Make up lost weight and adjust the reaction to a faint pink with phenol red, or if the phenolphthalein titration is used, and the reaction is not already between +0.5 and +1, adjust to +1.

4. Cool to 25° C. and filter through paper until clear.

5. Distribute in test-tubes, 10 c. c. to each tube.

6. Sterilize in the autoclave at 15 pounds (120° C.) for 15 minutes after the pressure reaches 15 pounds.

D. Media for Indol Test. (A. P. H. A., 1920). To 1,000 c. c. of distilled water add 0.3 gram tryptophane, 5 grams dipotassium hydrogen phosphate (K_2HPO_4), and 1 gram peptone. Heat until ingredients are thoroughly dissolved, tube (6 to 8 c. c.), and sterilize in autoclave for 15 minutes after the pressure reaches 15 pounds. Some American peptones are standardized to contain a uniform amount of tryptophane. If such peptone is used the tryptophane in the above formula may be omitted and the peptone increased to 5 grams.

E. Litmus Milk. (After Prescott and Winslow). The milk to be used as a culture medium shall be as fresh as possible, "Certified Milk" being ordinarily the best obtainable in city laboratories. It shall be placed

*The solution of the gelatin will be facilitated by allowing it to soak in the cold one-half hour before heating.

in a refrigerator over night to allow the cream to rise and the suspended matter to settle. The skimmed milk shall be siphoned off into a flask for use. It will be found more convenient, however, to allow the milk to stand in a separatory funnel. Should the milk be too acid the reaction shall be corrected to +1 by the addition of normal sodium hydrate. It is then ready to be tubed and sterilized. Litmus milk shall be prepared as above, with the addition of sterile 1 percent azolitmin. As it is impossible to make each lot of litmus milk with the same shade of color, it is recommended that a control tube be always exposed with the inoculated tubes for the purposes of comparison.

III. MEDIA FOR PRELIMINARY ENRICHMENT OR THE PRESUMPTIVE TEST.

A. Lactose Broth. (Standard Methods A. P. H. A., 1920). Sugar broths shall be prepared in the same general manner as nutrient broth with the addition of 0.5 percent of the required carbohydrate just before sterilization. The removal of muscle sugar is unnecessary as the beef extract and peptone are free from any fermentable carbohydrates. The reaction of sugar broths shall be a faint pink with phenol red or, if on titration with phenolphthalein the reaction is not already between neutral and +1, adjust to neutral. Sterilization shall be in the autoclave at 15 pounds (120° C.) for 15 minutes after the pressure reaches 15 pounds, provided the total time of exposure to heat is not more than one-half hour; otherwise a 10 percent solution of the required carbohydrate shall be made in distilled water and sterilized at 100° C. for 1½ hours, and this solution shall be added to sterile nutrient broth in amounts sufficient to make a 0.5 percent solution of the carbohydrate and the mixture shall then be tubed and sterilized at 100° C. for 30 minutes, or it is permissible to add by means of a sterile pipette directly to a tube of sterile neutral broth enough of the carbohydrate to make the required 0.5 percent. The tubes so made shall be incubated at 37° C. for 24 hours as a test for sterility.

B. Lactose (Peptone) Bile. The lactose bile medium consists of sterilized undiluted fresh ox gall (or a 10 percent solution of dry fresh ox gall) to which has been added 1 percent of peptone and 1 percent of lactose. The addition of peptone is important.

C. Lactose Bile Salt Broth. (After Savage).

Sodium taurocholate................5 grammes
Lactose ....5 grammes
Peptone ....20 grammes
Water ....1000 c. c.

These constituents are heated together until the solids are dissolved. The mixture is filtered, and sufficient neutral litmus solution is added to give a distinct color. The medium is then distributed into Durham's fermentation tubes and sterilized by steaming for twenty minutes on three successive days.

The sodium taurocholate prevents the growth of many saprophytic bacteria.

The presence of fermenting organisms, including *B. coli* is shown when the medium turns red (due to acid production) and gas is formed in the inner tube.

D. Glucose Broth. Same as lactose broth, substituting glucose for the disaccharid.

E. Liver Broth. (After Prescott and Winslow). 1. This medium is made from a hot infusion of beef liver instead of fresh meat, and is, in other respects, with the exception that phosphate is added the same as dextrose broth, but it is a richer food medium for bacteria. It gives gas formation with all species which ferment dextrose and develops attenuated bacteria, whether gas-forming or not, to a better degree than does beef broth. It is also especially suited to the rejuvenation of species in pure culture.

Formula

Beef Liver	500 gm.
Peptone	10 gm.
Dextrose	10 gm.
Di-Potassium Phosphate (K_2HPO_4)	1 gm.
Water	1000 gm.

2. Chop 500 gm. of beef liver into small pieces and add 1000 c. c. of distilled water. Weigh the infusion and container.

3. Boil slowly for 2 hours in a double boiler, starting cold and stirring occasionally.

4. Make up any loss in weight by evaporation and pass through a wire strainer.

5. To the filtrate add 10 gm. of peptone, 10 gm. of dextrose and 1 gm. of potassium phosphate.

6. After warming this mixture in a double boiler and stirring it for a few minutes to dissolve the ingredients, titrate with N/20 sodium hydrate, using phenolphthalein as an indicator, and neutralize with normal sodium hydrate.

7. Boil vigorously for 30 minutes in a double boiler, and 5 minutes over a free flame with constant stirring to prevent the caramelization of the dextrose.

8. Make up the loss in weight by evaporation and filter through cotton flannel and filter paper.

9. Tube and sterilize in an autoclave for 15 minutes at 120° C. (15 pounds).

The following media have been suggested for the elimination of spurious presumptive test:

F. Gentian Violet Lactose Broth. (Hall and Ellefson). The medium consists of 1 percent lactose broth containing 1-20,000 gentian violet.

G. Brilliant Green Lactose Bile (Muer and Harris). The composition of the medium used is as follows:

Distilled water	1,000 grams
Ox gall (dried)	50 grams
Peptone	10 grams
Lactose	10 grams
Brilliant-green	0.1 grams

Directions for Preparation

1. Heat 1 liter of distilled water in double boiler until water in outer vessel boils.
2. Add 50 grams of dried ox gall and 10 grams of peptone, stirring until all ingredients are dissolved.
3. Continue boiling for one hour.
4. Remove from flame and add 10 grams of powdered lactose.
5. Filter through cotton flannel until clear.
6. To each liter of the filtrate add 10 c. c. of a 1 percent solution of brilliant-green.
7. Tube and sterilize in autoclave for 15 minutes at 15 pounds pressure.

IV. MEDIA FOR DIRECT ISOLATION OR CONFIRMATION OF PRESUMPTIVE TEST.

A. Litmus Lactose Agar. (Wurtz Agar). (Standard Methods A. P. H. A., 1920).

Litmus-lactose-agar shall be prepared in the same manner as nutrient agar with the addition of 1 percent of lactose just before sterilization. The reaction shall be a faint pink with phenol red, or, if on titration with phenolphthalein the reaction is not already between neutral and +1, adjust to neutral. One c. c. of sterilized litmus or azolitmin solution shall be added to each 10 c. c. of the medium just before it is poured into the petri dish, or the mixture may be made in the dish itself.

B. Fuchsin Sulphite (Endo) Agar. Endo agar consists of nutrient lactose agar containing basic fuchsin decolorized with sodium sulphite as an indicator. Many modifications have been described. Those more commonly employed and method of use are listed below:

(a). Endo's Medium (Standard Methods A. P. H. A., 1920)

1. Add 5 grams of beef extract, 10 grams of peptone and 30 grams of agar dried for one-half hour at 105° C. before weighing, to 1,100 c. c. of distilled water. Boil on a water bath until all the agar is dissolved and then make up the loss by evaporation.
2. Cool the mixture to 45° C. in a cold water bath, then warm to 65° C. in the same bath without stirring.
3. Make up lost weight, titrate, and if the reaction is not already between neutral and +1, adjust to neutral.
4. Filter through cloth and cotton until clear.
5. Distribute 100 c. c. or larger known quantities in flasks large enough to hold the other ingredients which are to be added later.

6. Sterilize in the autoclave at 15 pounds (120° C.) for 15 minutes after the pressure reaches 15 pounds.

7. Prepare a 10 percent solution of basic fuchsin in 95 percent alcohol, allow to stand 20 hours, decant and filter the supernatant fluid. This is a stock solution.

8. When ready to make plates melt 100 c. c. of agar in streaming steam or on a waterbath. Dissolve 1 gram of lactose in 15 c. c. of distilled water, using heat if necessary. Dissolve 0.25 gram anhydrous sodium sulphite in 10 c. c. water. To the sulphite solution add 0.5 c. c. of the fuchsin stock solution. Add the fuchsin-sulphite solution to the lactose solution and then add the resulting solution to the melted agar. The lactose used must be chemically pure and the sulphite solution must be made up fresh.

9. Pour plates and allow to harden thoroughly in the incubator before use.

(b) Endo's Medium (Hygienic Laboratory Modification). 1. The Hygienic Laboratory-Endo medium consists of a 3 percent agar which is titrated and corrected to +0.5 to phenolphthalein, to which is added 3.7 cubic centimeters of a 10 percent solution of anhydrous sodium carbonate per liter. For convenience it is flasked, sterilized, and stored in 200 cubic centimeter quantities. When ready to use, the following ingredients are added to 200 cubic centimeters of agar as follows:

2. Dissolve 2 grams C. P. lactose in 25 to 30 cubic centimeters of distilled water, with the aid of gentle heat.

3. Dissolve 0.5 gram of anhydrous sodium sulphite in 10 to 15 cubic centimeters of distilled water.

4. To the sulphite solution add 1 cubic centimeter of saturated solution of basic fuchsin in 95 percent alcohol.

5. Add the fuchsin-sulphite solution to the lactose solution, and then add the whole to the agar. Pour plates at once and, after hardening, dry for 15 minutes in the incubator.

(c) Endo's Medium (Med. Dept. U. S. Army Modification).

1. Into a container put 1 liter of tap water, marking the level of the fluid. Add 30 grams of thread agar, 10 grams of peptone, 5 grams of NaCl, 5 grams of beef extract. Cook until dissolved—it is best to autoclave thirty minutes at 15 pounds; filter through sterile gauze or cotton. If necessary clear with egg. For this purpose, for each liter beat up the white of one egg with 10 c. c. of warm water until the egg is well mixed. Add this to agar cooled to 55° C., mix thoroughly, heat for 30 minutes or autoclave and filter through cotton.

2. This stock agar is kept on hand in quarter-liter flasks or bottles. Agar is standardized just before use and reaction adjusted to 0.2 percent acid to phenolphthalein. Before use, fuchsin and sodium sulphite are added. A filtered, saturated solution of basic fuchsin in 95 percent alcohol is kept on hand. A 10 percent solution of dry sodium sulphite crystals in sterile water is freshly made.

3. Teague has shown that a 10 percent solution of crystalline sodium sulphite can be heated for twenty minutes at 15 pounds pressure with practically no change, and that the 10 percent sodium sulphite solution covered with a layer of liquid petroleum about one cm. thick and sterilized in the autoclave can be kept at room temperature for three weeks and probably much longer with but very slight change.

4. One and eight-tenths c. c. of fuchsin solution is added per liter to the agar. After this has been done the sodium sulphite solution is added gradually until the hot agar is almost decolorized—usually about 25 c. c. to the liter. A pale rose color should be present in the hot agar, which fades to a very faint pink on cooling; 10 grams of lactose is dissolved in a little water, filtered and added to each liter.

Various fuchsin solutions may differ and the absolute quantities given above may not be exactly the proper balance in separate lots. These are approximate, however, and the proper balance can easily be attained by a little preliminary testing in which sodium sulphite solution is added to small quantities of fuchsin solution in a test-tube.

The finished product is poured into large sterile Petri dishes. The cover is left off until the agar is hard. Smears are made on these plates.

It is helpful to lay a piece of filter paper into the lid of the petri plate in order to absorb liquid evaporating from the agar in the incubator. If there is not enough filter paper for this, the plate should be placed upside down in the incubator.

(d) Endo's Medium (Kendal's Modification). **(1) Preparation of Agar.**—(a) Prepare plain, sugar-free nutrient agar, using 15 grams of agar per liter.

(b) Adjust the reaction to a point just alkaline to litmus.

(c) Flask the agar, 100 c. c. to a flask, and sterilize in the autoclave.

(2) Preparation of Indicator—(a) Prepare a 10 percent solution of basic fuchsin in 96 percent alcohol. This solution is fairly stable if kept away from light.

(b) Prepare a 10 percent aqueous solution of chemically pure anhydrous sodium sulphite (1 gram in 10 c. c. water). This solution does not keep.

(c) Add 1 c. c. of "2, a" to 10 c. c. of "2, b" and heat in the Arnold sterilizer for 20 minutes. The color of the fuchsin is nearly discharged if the solutions are of proper strength. This solution must be prepared each day—it does not keep.

(3.) Preparation and Use of Endo medium—(a) Add 1 gram of C. P. lactose (free from dextrose) to 100 c. c. of agar and place in the autoclave until melted and the lactose is thoroughly dissolved.

(b) Add a sufficient volume of "2, c" (about 1 c. c.) to impart a faint pink color to the medium.

(c) Pour into sterile Petri dishes and allow to harden in a dark place with the covers partly removed. When cool the medium should be colorless when viewed from above and a very faint pink when viewed from the

edge. The medium must be kept in a dark place because the color is restored by the action of daylight.

(e) **Endo's Medium** (Robinson and Rettger's Modification).

Water	1,000 c. c.
Agar (powdered)	25 grams
Peptone (American brand)	10 grams
Meat extract (Liebig's)	5 grams
Sodium carbonate (10% sol.)	10 c. c.
Lactose, c. p.	10 grams
Fuchsin (sat, alcoh. sol.)	5 c. c.
Anhydrous sodium bisulphite(10% sol.)	10 c. c.

Dissolve the agar, meat extract, and peptone. Make neutral to litmus paper, steam in the autoclave at 12 to 15 pounds extra pressure for 35 to 40 minutes, filter through absorbent cotton and cheesecloth, add the sodium carbonate solution, and heat for about 10 minutes in a boiling water bath. Introduce the lactose and the fuchsin into the hot liquid. The medium will now be brilliant red. Finally add the bisulphite solution. The hot medium is light red in color, is filled into large test-tubes, 20 cubic centimeters in each tube, and sterilized for five to seven minutes at 10 pounds extra pressure. When the medium has cooled completely it should be of a light pink or flesh color in the tubes, but transparent and practically colorless in the large Petri dishes. The tubed agar may be kept for several weeks in the refrigerator.

(f) **A Simplified Endo's Medium** (Levine) The medium is prepared as follows:

Distilled Water	100 c. c.
Peptone (Difco)	10 grams
Dipotassium phosphate (K_2HPO_4)	2 to 5 grams
Agar	15 to 30 grams

The ingredients are boiled until dissolved and any loss due to evaporation is made up with distilled water.

No adjustment of reaction is made and filtration is not necessary if the medium is to be used for streak plate cultures.

Measured quantities are placed in flasks or bottles and sterilized for 15 minutes at 15 pounds.

For use, the agar prepared as above is melted and the following materials added to each 100 c. c. of medium.

20 percent lactose solution	1 gram or 5.0 c. c.
10 percent (saturated) alcoholic solution of basic fuchsin	0.5 c. c.
Freshly prepared 10 percent sodium sulphite solution	2.5 c. c.

Plates are poured, allowed to harden in the incubator, and inoculated in the ordinary way.

(g) **Fuchsin (Endo) Agar** (Savage's Modification). 1. Peptone, 10 grams; Liebig's extract of beef, 10 grams; sodium chlorid, 5 grams, are boiled up in an enamelled dish with 1 liter of distilled water. The mixture is then poured into a flask, 30 grams of powdered agar added, and the whole heated in the autoclave at 115° C. for one hour. The flask is

removed, and, after cooling to about 60° C., the white of one egg mixed with a little distilled water is added. The contents are coagulated by heating in current steam in the usual way, filtered, and the filtrate made up to 1 liter. The mixture is made neutral, litmus paper being used as the indicator. Then 19 c. c. of **normal** sodium carbonate solution and 10 grams of chemically pure lactose are added. The flask is replaced for 30 minutes in the steam sterilizer. Almost invariably there is a considerable precipitate, and the mixture has to be again filtered.

2. Seven c. c. of the fuchsin solution (see below) are added, followed by 25 c. c. of a quite freshly prepared 10 percent sodium sulphite solution. The mixture becomes much less red, but is not immediately decolorized. It is then tubed, conveniently into small flasks, each containing 50 to 60 c. c. of media, and sterilized in current steam for two days, 30 minutes each day.

3. The fuchsin solution is made as follows: Three grams of powdered crystalline fuchsin are placed in a dry flask, and 60 c. c. of absolute alcohol are added. The contents are thoroughly mixed, and the flask, tightly stoppered, allowed to stand for exactly 24 hours at 20° to 22° C. The alcoholic extract is then decanted and preserved in a clean glass-stoppered bottle. Made in this way a uniform fuchsin extract is obtained which keeps well, and the same quantity of fuchsin is added each time a fresh batch of medium is prepared; a matter of much importance.

The medium must be stored in the dark, since light gradually turns it red. When solidified it is almost free from color.

(h.) Conradi-Drigalski Agar (After Prescott and Winslow) These authors have modified lactose litmus agar by adding to it nutrose and crystal violet and by using three percent of agar instead of one percent. The crystal violet strongly inhibits the growth of many other bacteria, especially cocci, which would also color the medium red; the 3 percent agar makes the diffusion of the acid which is formed more difficult.

Three pounds of chopped beef are allowed to stand 24 hours with two liters of water. The meat infusion is boiled one hour and filtered. Twenty gm. of Witte's peptone, 20 gm. of nutrose, and 10 gm. of NaCl are then added, and the mixture boiled another hour. After filtration and the addition of 60 gm. of agar the mixture is boiled for three hours, made alkaline and filtered. In the meantime 300 c. c. of litmus solution (Kahlbaum) are boiled for 15 minutes with 30 gm. of lactose. Both solutions are then mixed and the mixture, which is now red, made faintly alkaline with 10 percent soda solution. To this feebly alkaline mixture 4 c. c. of hot sterile 10 percent soda solution are added and 20 c. c. of a sterile solution (1 to 1000) of crystal violet (Hochst B.).

(i) Bile Salt (Rebipel) Agar (After Savage). Sodium taurocholate 5 grams, Witte's peptone 20 grams, and distilled water 1 liter, are boiled up together, 20 grams of agar are added and dissolved in the solution in the autoclave in the ordinary way. The medium is cleared with white of egg and filtered. After filtration, 10 grams of lactose and 5

c. c. of recently prepared 1 percent neutral red solution are added. The medium is then tubed and sterilized for 15 minutes on three successive days.

(j) Aesculin Agar. (After Eyre) (B. coli and allied organisms give black colonies surrounded by black halo.)

Measure out 400 c. c. distilled water into a tared 2-liter flask.

Weigh out

Agar ....15 grams
Peptone ....10 grams
Sodium taurocholate .... 5 grams

and make into a thick paste with 150 c. c. distilled water.

Add this paste to the distilled water in the flask.

Dissolve the ingredients by bubbling live steam through the mixture.

Weigh out

Aesculin ....1.0 gram
Ferric citrate ....0.5 gram

and dissolve in a second flask containing 100 c. c. distilled water.

Mix the contents of the two flasks—adjust the weight to the calculated medium figure (in this case 1031.5 grams) by the addition of distilled water at 100° C.

Clarify with egg and filter.

Tube and sterilize as for nutrient agar.

(k) A Simplified Eosine Methylene Blue Agar. (Levine)

Distilled water ....1000 c. c.
Peptone (Difco) .... 10 grams
Dipotassium phosphate (K_2HPO_4)........... 2 grams
Agar .... 15 grams

Boil ingredients until dissolved and make up any loss due to evaporation with distilled water.

Place measured-quantities (100 or 200 c. c.) in flasks or bottles, and sterilize in the autoclave at 15 pounds pressure for 15 to 20 minutes.

Just prior to use add, to each 100 c. c. of the melted agar prepared as above, the following:

Lactose, sterile 20% solution........5 c. c. or 1 gram dry substance
Eosine yellowish 2% aqueous sol.................................2 c. c.
Methylene-blue 0.5% aqueous sol.................................2 c. c.

Pour medium into perti dishes, allow them to harden, and inoculate by streaking on the surface.

There is no adjustment of the reaction and filtration of the medium is not necessary.

V. SPECIAL MEDIA FOR DIFFERENTIATION OF THE COLI AND AEROGENES SECTIONS.

A. Clark and Lubs Medium. (Standard Methods A. P. H. A., 1920). 1. To 800 c. c. of distilled water add 5 grams of Proteose-Peptone, Difco., or Witte's Peptone (other peptones should not be substituted), 5 grams c. p. dextrose, and 5 grams dipotassium hydrogen phosphate

(K_2HPO_4). A dilute solution of the K_2HPO_4 should give a distinct pink with phenolphthalein.

2. Heat with occasional stirring over steam for 20 minutes.
3. Filter through folded filter paper, cool to 20° C. and dilute to 1,000 c. c. with distilled water.
4. Distribute 10 c. c. portions in sterilized test tubes.
5. Sterilize by the intermittent method for 20 minutes on three successive days.

B. Synthetic Medium. (After Clark and Lubs). (Standard Methods A. P. H. A., 1920).

1. Na_2HPO_4 (anhydrous) .... 7 grams
or
$Na_2HPO_4 . 2H_2O$ .... 8.8 grams
KHphthalate .... 2 grams
Asparatic acid .... 1 gram
Dextrose .... 4 grams
Warm distilled water .... 800 c. c.

2. When solution is complete, cool and make up to 1 liter at room temperature.
3. Heat in an autoclave for 15 minutes after the pressure has reached 15 pounds, provided the total time of exposure to heat is not more than one-half hour.
4. The hydrogen-ion concentration of the medium is fixed by the composition. It should be very close to PH 7.0, slightly red with phenol red. All materials should be re-crystallized or if used from stock furnished by manufacturers, should be carefully examined. The di-sodium hydrogen phosphate may be used either as the anhydrous salt obtained by dessication in vacuo at 100° C. or else as the salt containing two molecules of water of crystallization. This is obtained by exposing the re-crystallized $Na_2HPO_4 12H_2O$ for two weeks. Use 0.88 percent of Na_2HPO_4 $2H_2O$.

C. Uric Acid Medium (Koser)

Distilled ammonia-free water .... 1,000 c. c.
NaCl .... 5.0 gm.
MgSO4 .... 0.2 gm.
$CaCl_2$ .... 0.1 gm.
K_2HPO_4 .... 1.0 gm.
Glycerol .... 30.1 gm.
Uric acid .... 0.5 gm.

This combination gives a colorless and clear medium. It is filled into ordinary test tubes and sterilized in the autoclave at 13 to 15 pounds extra pressure for 15 minutes. A slight turbidity may be apparent after autoclaving, due, presumably, to a finely divided precipitate of calcium sulphate. On cooling, the solution becomes clear.

On the addition of 1.5 percent of washed shred agar to the solution mentioned in the foregoing an agar medium was obtained on which the same distinction between the two types may be brought out.

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